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## Activation and inhibition of GTPase translation factors on the prokaryotic ribosome

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**Activation and Inhibition of GTPase  
Translation Factors on the Prokaryotic Ribosome**

By

Justin D. Walter

Accepted in Partial Completion  
Of the Requirements for the Degree  
Master of Science

Moheb A. Ghali, Dean of the Graduate School

ADVISORY COMMITTEE

Chair, Dr. P. Clint Spiegel

Dr. Spencer Anthony-Cahill

Dr. Gerry Prody

## **MASTER'S THESIS**

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Justin D. Walter  
November 23, 2010

**Activation and Inhibition of GTPase**  
**Translation Factors on the Prokaryotic Ribosome**

A Thesis  
Presented to  
The Faculty of  
Western Washington University

In Partial Fulfillment  
Of the Requirements for the Degree  
Master of Science

By  
Justin D. Walter  
November 2010

## **Abstract**

Throughout all domains of life, each protein in a cell is synthesized by a remarkable biomolecular machine called the ribosome, in a process referred to as translation. This process is regulated by proteins called translation factors, several of which belong to the GTPase superfamily of enzymes which require the binding and subsequent hydrolysis of guanosine 5'-triphosphate (GTP) to execute their function. In contrast to the regulatory role of translation factors, protein biosynthesis is inhibited by several naturally occurring antibiotics. While our understanding of translation has been revolutionized by the recent elucidation of atomic-resolution x-ray crystal structures of the ribosome trapped in various intermediate conformations, several crucial aspects of protein biosynthesis remain poorly understood, such as the identity of the molecular component of the ribosome which stimulates the activation of the translational GTPases, as well as the mechanism by which several antibiotics inhibit translation.

The major aims of this work are twofold. First, investigations directed towards the elucidation of the ribosomal element responsible for GTPase activation are described. It is demonstrated that the depletion of a specific protein from the ribosome which is part of the GTPase binding site, L12, results in significant attenuation of ribosome-dependent GTP hydrolysis activity by translational GTPases IF2, EF-G, LepA, and RF3, and this lost activity is fully restored by pre-

incubating L12-depleted ribosomes with purified L12 protein. However, L12 alone does not stimulate GTP hydrolysis by these GTPases, in contrast to a previous report (Savelsbergh et al., 2000). In fact, it is shown that none of the isolated rRNA or protein components which comprise the ribosomal GTPase binding region stimulate GTP hydrolysis by the translational GTPases, implying that the peripheral ribosomal architecture is needed for correct positioning of the GTPase-activating element of the ribosome.

A second major goal of this work was to investigate the inhibitory mechanism of the antibiotic thiostrepton, which is known to interfere with the function of elongation factor EF-G, and has been recently shown to inhibit the growth of the malarial parasite *Plasmodium falciparum*. Many lines of evidence reported herein contradict the current predominantly accepted model of thiostrepton action. It is shown that thiostrepton strongly inhibits ribosome-dependent GTP hydrolysis by EF-G and a closely related GTPase LepA, and this is explained by results which indicate that thiostrepton obstructs the binding of these factors to the ribosome. Interestingly, an engineered mutant of EF-G lacking domains IV and V is insensitive to thiostrepton, which is in agreement with recent structural evidence which suggests that thiostrepton interferes with the interaction between domain V of EF-G and the ribosome.

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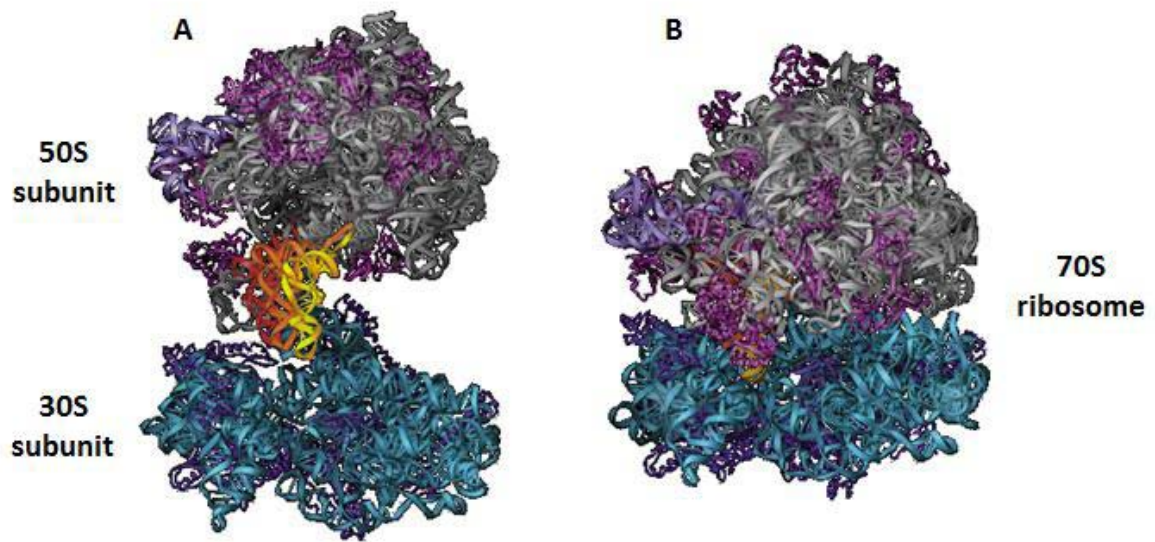
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## Chapter 1 – The ribosome, translation, and GTPases

The “central dogma” of molecular biology describes how genetic information flows from a DNA storage medium (genes), through an RNA intermediate (messenger RNA), and, ultimately, into a functionally relevant form (protein) (Crick, 1970). The final step in this pathway, the biosynthesis of protein based on a specific sequence of messenger RNA (mRNA), is mediated by a large biomolecular complex called a ribosome (Figure 1-1). In essence, the ribosome serves as the crucial biological link between the genotype (genetic endowment) and phenotype (physical attributes) of an organism. Specifically, ribosomes catalyze the sequential, ordered formation of peptide bonds between amino acids, in an exact sequence which is directed by mRNA, mediated by transfer RNAs (tRNAs), and controlled by a variety of regulatory proteins. This process is referred to as translation, due to the fact that the four-variable nucleotide language of DNA and RNA is *translated* into the 20-variable amino acid language of proteins. The precise association of a single amino acid with a specific mRNA sequence element called a codon is the basis of the genetic code (Crick, 1968), and the ubiquity of this code throughout the domains of life suggests that this translational system must be one of the most evolutionarily ancient biological processes (Osawa et al., 1992; Woese et al., 1966).

## **Prokaryotic ribosome structure**

Prokaryotic ribosomal architecture consists of a complex foundation of both RNA and protein. The ribosome is composed of two independent, asymmetric subunits (Figure 1-1). The large, or 50S, subunit is composed of two strands of RNA, denoted 23S and 5S rRNA (2900 and 120 nucleotides in length, respectively) and 33 ribosomal proteins (named L1, L2... etc). The small, 30S subunit is composed of one RNA strand of approximately 1500 nucleotides, denoted the 16S strand, and 21 ribosomal proteins (S1, S2... etc) (Wilson and Nierhaus, 2003). The 16S rRNA is the site of mRNA binding, and tRNAs recognize the full 70S•mRNA complex. The core structural configuration of each ribosomal subunit is predominantly determined by rRNA, while ribosomal proteins are believed to have appeared later in ribosome evolution and generally appear to have more peripheral structural roles (Yusopov et al., 2001). Altogether, the assembled 70S ribosome complex has a molecular weight of approximately  $2.5 \times 10^6$  Da and a diameter of roughly 250 Å (Ramakrishnan, 2002; Wilson and Nierhaus, 2003). Remarkably, although there are notable differences in particular details of ribosome structure between species, all critical regions of the ribosome bear a remarkable degree of conservation in sequence and structure, suggesting that many of the core processes such as tRNA recognition, peptide bond formation, and translocation, are likely to follow similar mechanisms across all the domains of life (Ganoza et al., 2002; Ramakrishnan, 2002).

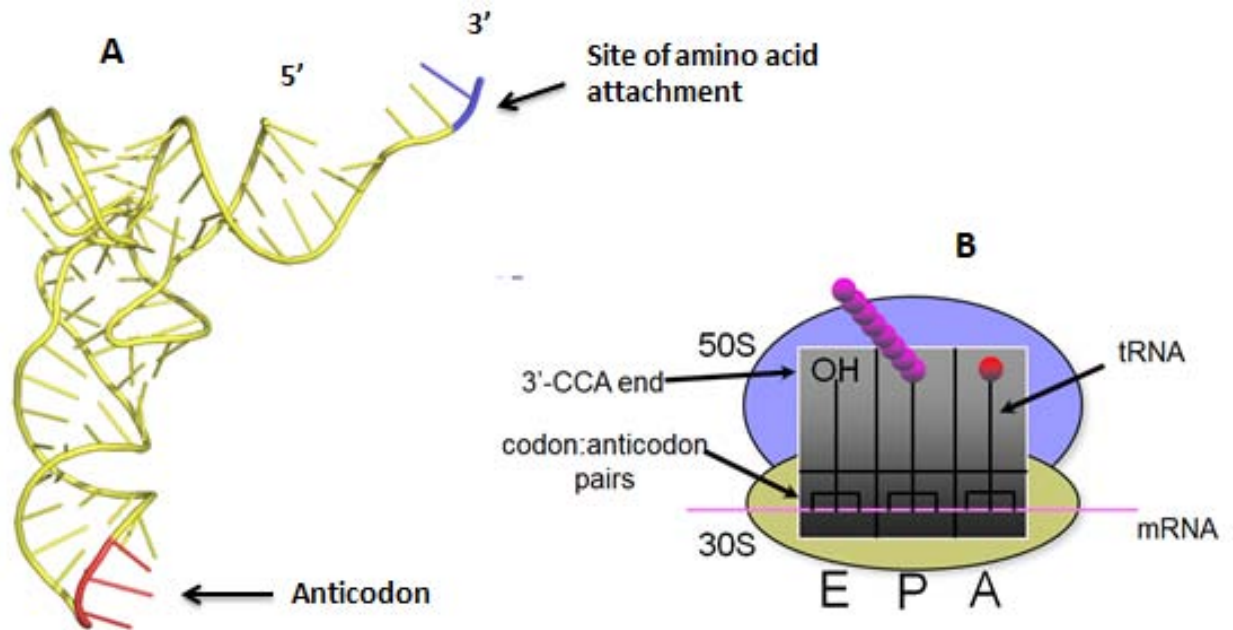


**Figure 1-1. Crystal structure of the ribosome at 5.5 Å resolution. (A) Individual subunits. (B) 70S complex. 50S subunit: Grey, 23S rRNA; Purple, ribosomal proteins. 30S subunit: Cyan, 16S rRNA; Blue, ribosomal proteins. E, P, and A-site tRNAs are red, orange, and yellow, respectively. (Yusupov et al., 2001).**

### *Transfer RNAs*

Transfer RNA molecules (Figure 1-2) serve as the “adaptors” which recognize a specific mRNA sequence element called a codon and deliver the corresponding amino acid to the ribosome. There are three tRNA binding sites on the ribosome, the A site, the P site, and the E site (Agrawal et al., 1996; Rheinberger et al., 1981). The A site binds aminoacyl-tRNA (aa-tRNA), that is, a tRNA bound to a single amino acid. The P site binds peptidyl-tRNA, which is a tRNA molecule bound to the growing nascent polypeptide chain. Finally, the E site binds

deacylated, or “uncharged” tRNAs (deacyl-tRNAs) which are a product of peptide elongation on the ribosome.



**Figure 1-2. (A) Crystal structure deacyl-tRNA<sup>Phe</sup>, indicating the anticodon region as well as the site of amino acid attachment; (PDBid 2WRI). (B) Schematic representation of the 70S ribosome, indicating the three tRNA binding sites.**

## Translation

Functionally, the ribosome is a polymerase which, as specified by the sequence of codons on a strand of mRNA, catalyzes the formation of peptide bonds between amino acids to synthesize a protein. This process, translation, proceeds in four distinct stages: initiation, elongation, termination, and recycling (Table 1). Within each of these stages, there are proteins called translation factors which transiently interact with the ribosome and regulate steps in the



translational cycle. As such, they are typically named in accordance with the particular stage of translation with which they are associated (i.e. *initiation* factor 2, *elongation* factor G, *release* factor 3, etc.).

Table 1. Stages of translation and associated translation factors

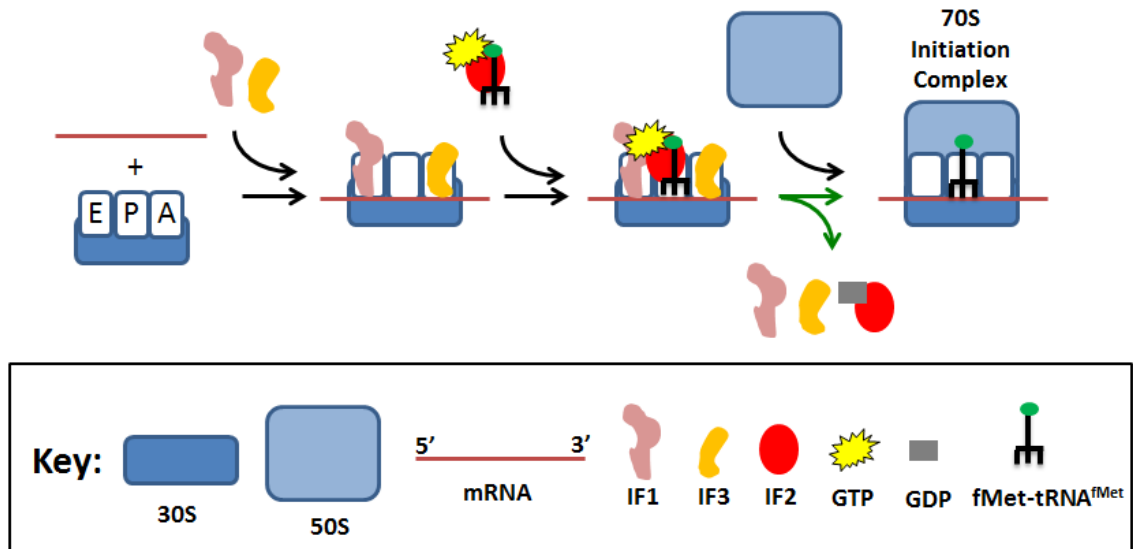
Translational Stage	Associated Translation Factors
Initiation	Initiation factors 1-3 (IF1, IF2, IF3)
Elongation	Elongation factors Tu and G (EF-Tu, EF-G)
Termination	Release factors 1-3 (RF1, RF2, RF3)
Recycling	Ribosome recycling factor (RRF), EF-G

### *Initiation*

Every prokaryotic protein sequence begins with N-formylmethionine (fMet), a modified methionine with a formyl group added to the amine portion (Salas et al., 1967). This initiator residue is generally removed from the peptide following the completion of protein biosynthesis (Ball and Kaesberg, 1973). The initiation stage of translation (Figure 1-3) involves the formation of an “initiation complex,” consisting of the 70S ribosome, a strand of mRNA, and an initiator tRNA (consisting of a tRNA molecule bound to fMet, denoted hereafter as fMet-tRNA<sup>fMet</sup>) bound to the ribosomal P site. To commence the initiation of protein

synthesis, mRNA binds spontaneously to the 30S subunit via specific hydrogen bonding interactions between the Shine-Dalgarno sequence, present upstream of the protein-coding region within all prokaryotic mRNA transcripts, and a conserved complementary region of 16S rRNA (Shine and Dalgarno, 1974). Binding of mRNA to the 30S subunit places a start codon, coding for fMet-tRNA<sup>fMet</sup>, directly in the P site. Subsequent steps of initiation involve three initiation factors, IF1, IF2, and IF3 (Gualerzi and Pon, 1990). IF1 binds to the 30S subunit near the A site, and is believed to play a role in preventing the premature entry of the next aminoacyl-tRNA to the ribosome during initiation of a polypeptide (Ramakrishnan 2002). IF2, a GTPase, is thought to bind to fMet-tRNA<sup>fMet</sup> and control its transfer to the 30S P site while preventing the binding of non-cognate tRNAs (the structure and mechanism of GTPases will be further discussed in detail later in this chapter) (Roll-Mecak et al., 2000). IF3 binds tightly to the 30S subunit near the E-site and prevents its premature association with the 50S subunit while the initiation complex is formed (Petrelli et al., 2001). Once initiator tRNA has been accommodated into the P site, IF3 dissociates from the ribosome via a largely undescribed mechanism. Finally, at some undetermined stage of initiation, guanosine 5'-triphosphate (GTP) is hydrolyzed by IF2, causing a conformational change which releases it from the complex and allows the full 70S initiation complex to form, poised for the elongation cycle. (Luchin et al., 1999). Although decades of biochemical and structural studies have shed light on the individual roles of the initiation factors, an unambiguous

description of the order in which the factors bind and are released in vivo remains elusive.



**Figure 1-3. The initiation stage of translation.** After the spontaneous association of mRNA with the 30S subunit, translation factors IF1, IF2•GTP•fMet-tRNA<sup>fMet</sup>, and IF3 mediate the formation of the 70S initiation complex. Steps with a green arrow involve GTP hydrolysis by the GTPase IF2.

### *Elongation*

The elongation phase is a cyclical process, and consists of three distinct steps which iteratively repeat, systematically lengthening the nascent peptide one amino acid at a time, until a termination codon is encountered: *accommodation* of the appropriate aminoacyl-tRNA into the A site, *peptidyl transfer*, and *translocation* of the mRNA•tRNA complex through the ribosome.

### *Elongation step 1 - Accomodation*

Upon completion of the initiation cycle, fMet-tRNA<sup>fMet</sup> is bound in the P site of the 70S ribosome and the A site is empty. Aminoacylated tRNA is carried to the A site via a ternary complex consisting of elongation factor Tu (EF-Tu) bound to GTP and aa-tRNA. Correct “decoding” of the A site mRNA codon involves the interaction between the complementary anticodon of the EF-Tu-bound aminoacyl tRNA (Pape et al., 2000; Schmeing et al., 2009). This results in stabilization of tRNA binding and subsequent conformational changes which cause GTP hydrolysis by EF-Tu (Figure 1-4) (Berchtold et al., 1993). This selection process, called accommodation, is followed by the dissociation of EF-Tu•GDP from the ribosome (Pape et al., 1998; Potapov, 1982).

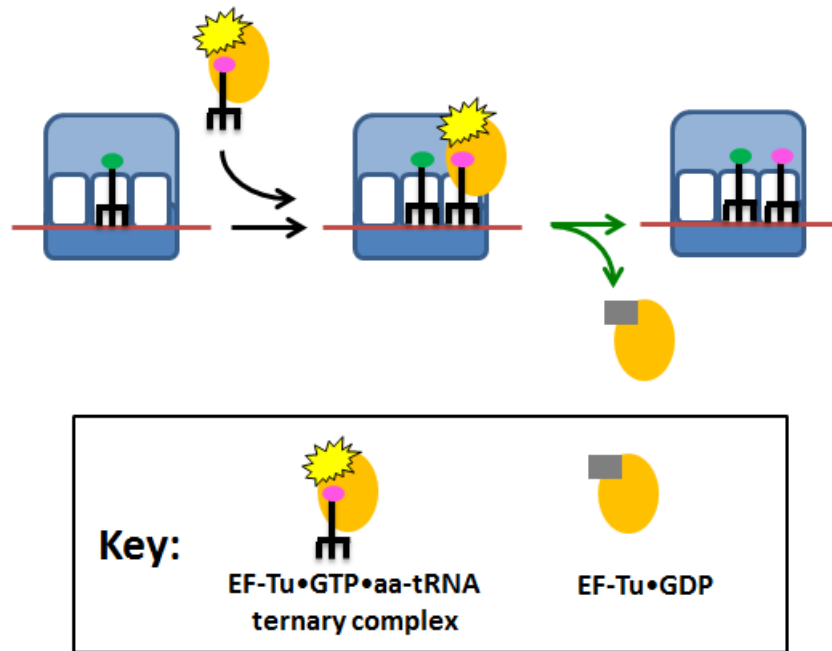
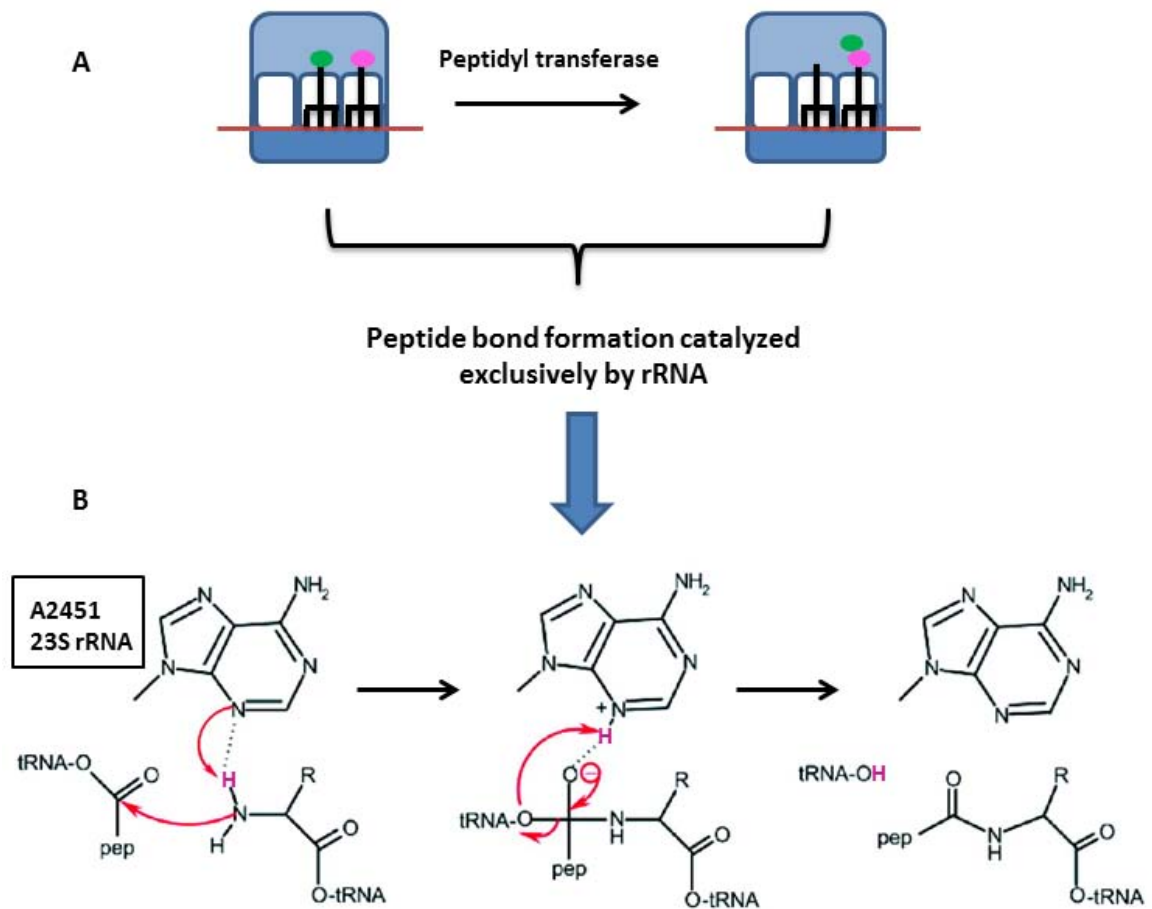


Figure 1-4. The accommodation step of elongation. Only the correct A site codon-anticodon pairing results in GTP hydrolysis and dissociation of EF-Tu from the ribosome. Green arrows indicate steps involving GTP hydrolysis.

### *Elongation step 2 - Peptidyl transfer*

Following the accommodation step, the aminoacyl end of the A site tRNA and the end of the P site tRNA harboring either fMet (directly after initiation) or the growing polypeptide chain, are ideally juxtaposed within the peptidyltransferase center (PTC) for peptide bond formation to occur (Figure 1-5). Peptide bond formation is catalyzed by a complex network of universally conserved rRNA and tRNA nucleotides (Nissen, 2000). A large milestone in the history of ribosome investigation was the discovery that it is exclusively RNA, not protein, which performs the catalytic duties of peptide bond formation. Specifically, the

catalyzed reaction involves a nucleophilic attack by the  $\alpha$ -amino group of the A site aminoacyl-tRNA on the ester which links the P site tRNA with the growing polypeptide (Nissen, et al. 2000). Many mechanisms for this step have been proposed, based on structural, biochemical, and computational experiments (Barta et al., 2001). One pioneering study suggested that the universally conserved nucleotide A2451 is oriented in such a manner which allows it to act as a general acid-base in the peptidyl transferase reaction, as indicated in Figure 1-5B (Nissen, et al. 2000). However, subsequent reports have challenged this proposed mechanism (Beringer et al., 2005; Beringer and Rodnina, 2007; Lang et al., 2008). Overall, this reaction accomplishes two necessary steps of elongation: the length of the peptide is increased by one amino acid, and the nascent peptide is transferred from the P-site tRNA to the A-site tRNA.



**Figure 1-5. The peptidyl transferase step of elongation. (A) The product of the peptidyl transferase reaction is an A-site peptidyl tRNA. (B) A proposed general acid-base mechanism for the reaction, involving the universally conserved rRNA nucleotide A2451. Adapted from Nissen et al., 2000.**

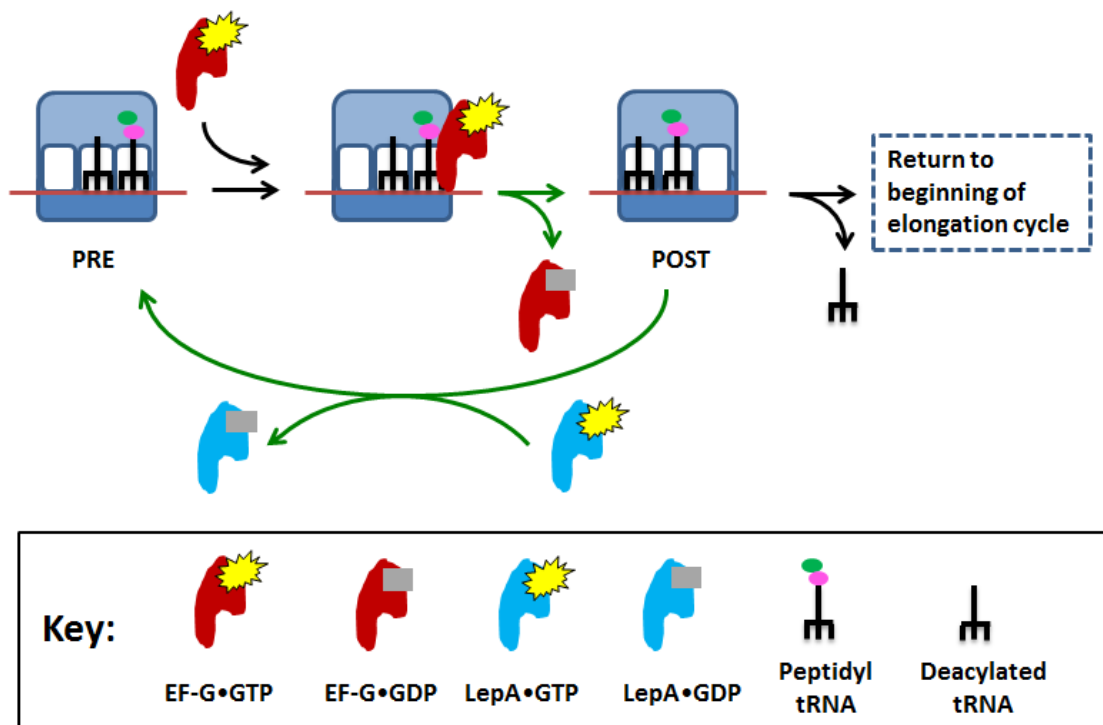
### *Elongation step 3 – Translocation*

After peptide bond formation, the peptidyl-tRNA in the A site and deacylated P site tRNA must be physically moved to the P and E sites, respectively, for the elongation cycle to continue. This process, called *translocation* (Figure 1-6), must precisely move the tRNA-mRNA complex in such a manner that the correct reading frame on mRNA is preserved, and it is catalyzed by the GTPase elongation factor G (EF-G). Binding of EF-G•GTP to the ribosome and subsequent GTP hydrolysis results in the coordinated movement of tRNAs and mRNA through the ribosome (Agrawal et al., 1999; Spiegel et al., 2007). At the end of translocation, deacylated tRNA dissociates from the E-site (Robertson and Wintermeyer, 1987; Spirin, 1984) and peptidyl-tRNA, now in the P site, is ready for the addition of another amino acid.

In contrast to the role of EF-G, a recently discovered GTPase translation factor, LepA (also called EF4), has been shown to catalyze the reverse translocation of tRNA and mRNA on the ribosome (Qin et al., 2006). LepA is highly conserved throughout bacterial species and is also found in the mitochondria of eukaryotes (Qin et al., 2006). With the exception of a novel C-terminal domain, its domain structure is remarkably similar to that of EF-G which suggests that it carries out its function in a manner similar to that of EF-G (Evans et al., 2008). Although LepA-catalyzed reverse translocation has been observed in vitro, its precise physiological role is unclear. It has been suggested that LepA acts to slow down



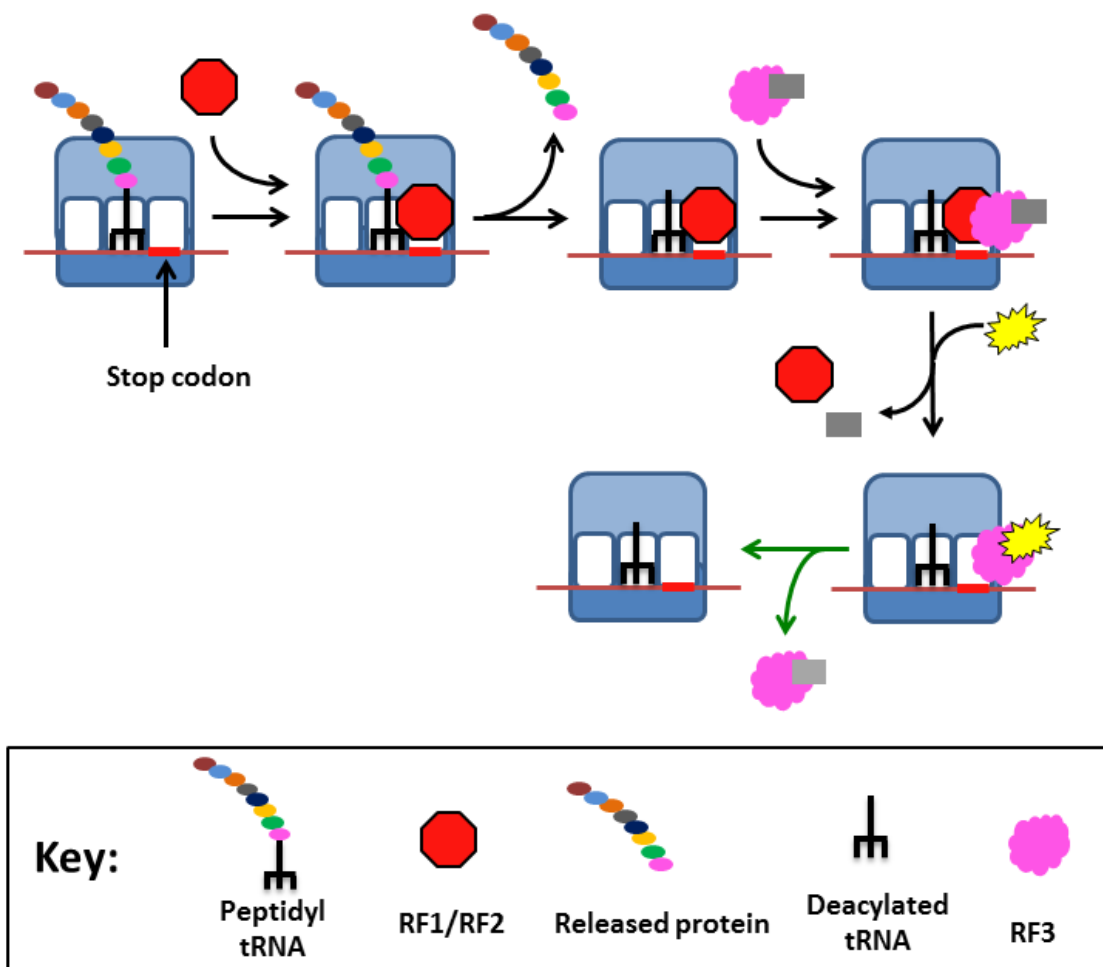
the rate of translation, which may contribute to the fidelity of protein folding and improve the active fraction of synthesized proteins (Qin, Polacek et al. 2006). An alternative hypothesis suggests that in suboptimal solution conditions such as high ionic strength, EF-G may not always promote complete translocation, and LepA could act to reverse this flawed translocation event, effectively offering a “second chance” for proper forward translocation. In any case, the role of LepA is poorly understood and additional experimentation will be necessary to elucidate its exact physiological function (Qin, Polacek et al. 2006).



**Figure 1-6. The translocation step of elongation.** EF-G catalyzes the forward translocation of the tRNA-mRNA complex from the pre-translocation state (PRE) to the post-translocation state (POST), while LepA catalyzes the opposite reaction. Green arrows indicate steps involving GTP hydrolysis.

### *Termination*

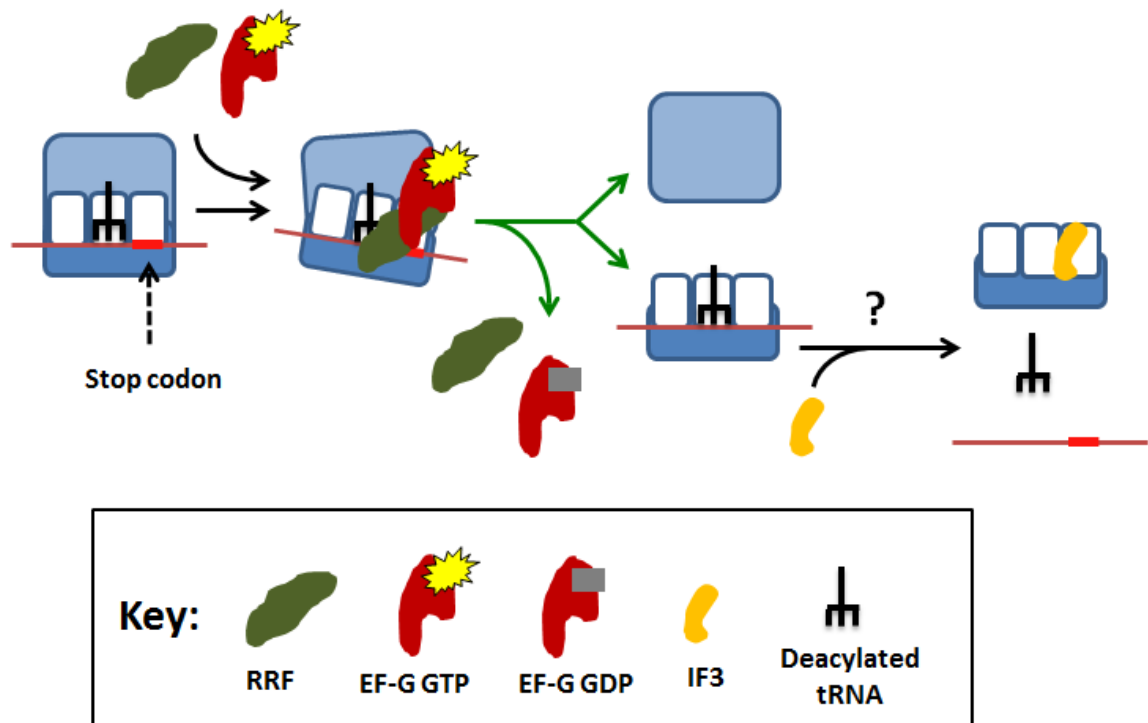
Following polypeptide elongation, the A site encounters a stop codon, and the process of termination occurs (Figure 1-7). The three possible stop codons are recognized by one of two “class I” peptide release factors, RF1 or RF2 (Kisselev et al., 2003). RF1 recognizes the UAG stop codon, RF2 recognizes UGA and both factors recognize UAA (Mora et al., 2003). Binding of a class I release factor results in hydrolysis and release of the nascent polypeptide from the P-site tRNA, although it is debated whether the role of RF1/2 in this reaction is direct or indirect (Petry et al., 2005; Rawat et al., 2006). Next, “class II” release factor RF3, which possesses GTPase activity, binds to the ribosome in complex with GDP. Once bound to the ribosome, GDP is replaced with GTP, causing a conformational change which results in the dissociation of RF1/2 from the ribosome (Gao et al., 2007). Finally, hydrolysis of GTP occurs, prompting the dissociation of RF3-GDP from the ribosome (Zavialov et al., 2002).



**Figure 1-7. Termination of protein biosynthesis.** Binding of a class I release factor (RF1/2) to a stop codon in the A site stimulates hydrolysis of the nascent peptide attached to a P site peptidyl-tRNA. Binding of the class II release factor RF3 in the GDP state dissociates RF1/2 from the ribosome. Finally, the ribosome stimulates GDP-GTP exchange, followed by GTP hydrolysis (indicated by green arrows) which results in dissociation of RF3-GDP from the ribosome.

## *Recycling*

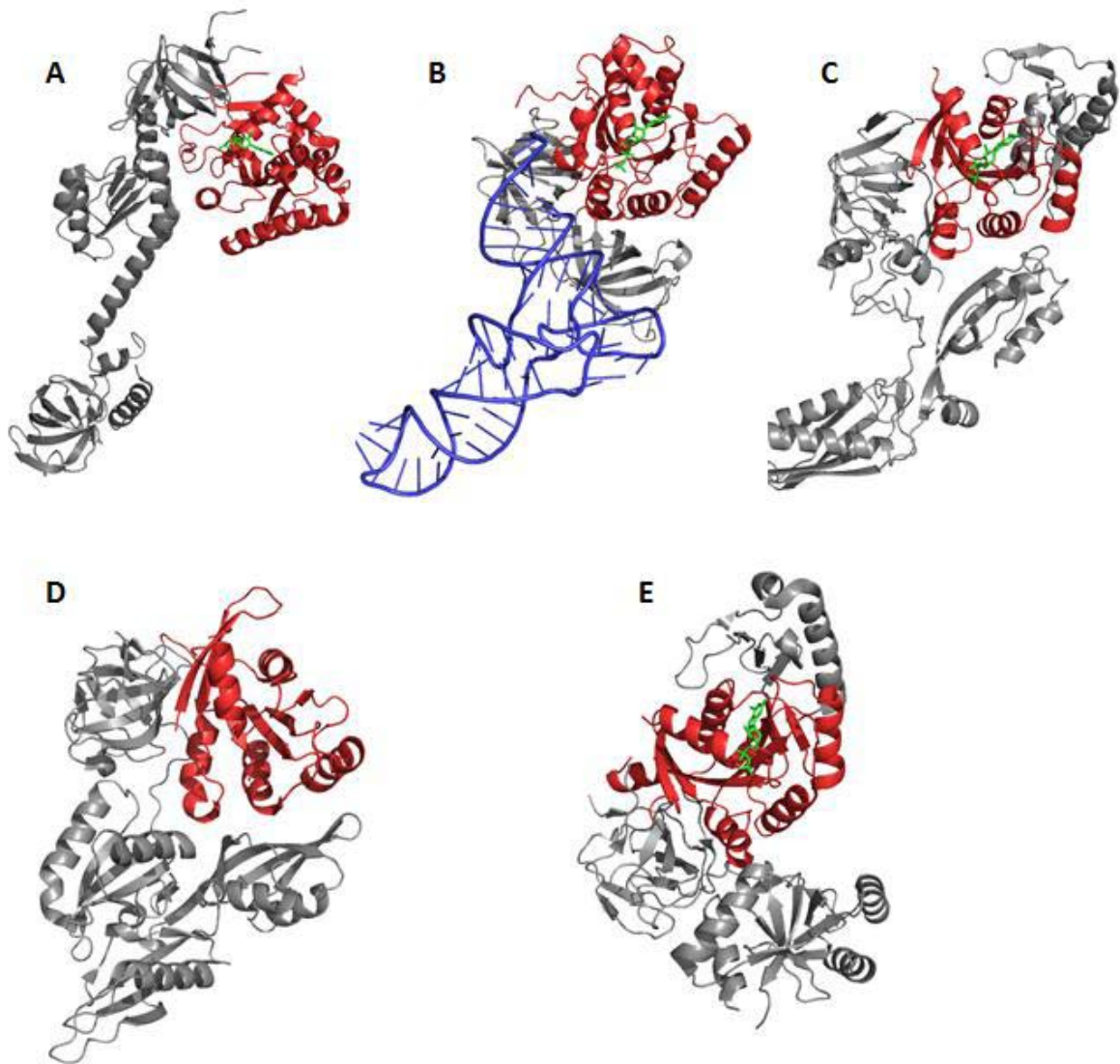
After the termination stage, mRNA and a deacylated P-site tRNA remain bound to the 70S ribosome. Before a new round of translation can occur, this post-termination complex must be completely disassembled. This task is accomplished by ribosome recycling factor (RRF), along with EF-G and IF3 (Figure 1-8), although the order of binding and relative contribution of each of these factors in the recycling process is debated (Hirokawa et al., 2008; Janosi et al., 1996). It is generally agreed that RRF and EF-G act together to dissociate the two ribosomal subunits (Peske et al., 2005). A crystal structure of RRF bound to the *Deinococcus radiodurans* 50S subunit revealed that the binding of RRF causes a significant rearrangement of an rRNA helix within the 50S subunit which forms a crucial bridge between the two ribosomal subunits (Wilson et al., 2005). However, RRF-induced disruption of intersubunit bridges was not observed in a crystal structure of RRF bound to the entire 70S ribosome (Weixlbaumer et al., 2007). It is proposed that the binding of EF-G to the RRF-70S complex possibly causes further movement of RRF which ultimately results in the dissociation of the ribosomal subunits (Wilson et al., 2005). Following subunit dissociation, it is thought that the binding of IF3 causes dissociation of mRNA and deacyl-tRNA, and prevents the re-association of the ribosomal subunits (Hirokawa et al., 2006). At this stage, the ribosome is ready to begin a new round of protein synthesis. Figure 1-8 provides an overview of this process.



**Figure 1-8. Ribosome recycling.** The binding of RRF destabilizes key intersubunit interactions, and subsequent binding and GTP hydrolysis by EF-G dissociates the ribosome into its 50S and 30S subunits. Dissociation of deacyl-tRNA and mRNA is thought to be mediated by IF3, but this step is not well understood. After the completion this phase, translation is complete.

### *Several translation factors are GTPases*

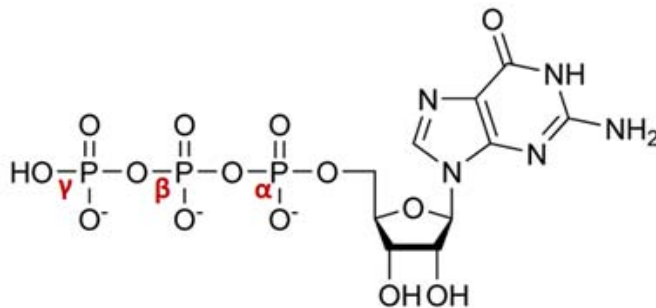
As noted above, many of the prokaryotic translation factors, IF2, EF-Tu, EF-G, LepA, and RF3, are GTPases. As with the other known GTPases, these proteins bind to GTP (Figure 1-10) via a consensus “G-domain” (Figure 1-11), and then harness the energy released upon hydrolysis of GTP to drive their respective regulatory functions. Due to this shared feature, it has long been proposed that these particular translation factors, albeit diverse in functional roles, have a common means of interaction with the ribosome (Moazed et al., 1988). In fact, the translational GTPases do indeed share an overlapping binding region on the ribosome, which will be discussed in detail momentarily. First, however, a thorough examination of the well conserved structural and functional details of GTPases will be presented.



**Figure 1-9. GTPase translation factors.** (A) IF2•GDP, PDBid 1G7S (Roll-Mecak, et al. 2000) (B) EF-Tu•GTP•Phe-tRNA<sup>Phe</sup> ternary complex, (Nissen et al., 1995); (C) EF-G•GDP, (Czworkowski et al., 1994); (D) LepA, (Evans et al., 2008); (E) RF3•GDP, (Gao et al., 2007) ; The conserved G-domain within each GTPase is colored red, structure not within the G domain is colored grey, guanine nucleotides are colored green, and Phe-tRNA<sup>Phe</sup> is colored blue.

## The GTPase superfamily

Proteins which belong to the GTPase superfamily are ubiquitous throughout the domains of life and regulate key steps in a wide variety of cellular processes, such as sensual perception (Wilkie, 1999), signal transduction (Bourne et al., 1991), and cell differentiation (Rossman et al., 2005), via a molecular “switch” which is activated or deactivated depending upon the identity of a bound guanosine nucleotide (Scheffzek et al., 1998). Typically, the binding of guanosine 5'-triphosphate (GTP, Figure 1-10) induces an active conformation, which is followed by the hydrolysis of the terminal  $\gamma$ -phosphate group of GTP to produce guanosine 5'-diphosphate (GDP). For example, in the case of the translational GTPase EF-G, the energy of GTP hydrolysis is believed to be converted into the directional molecular movement of tRNAs and mRNA through the ribosome (Rodnina et al., 1997).

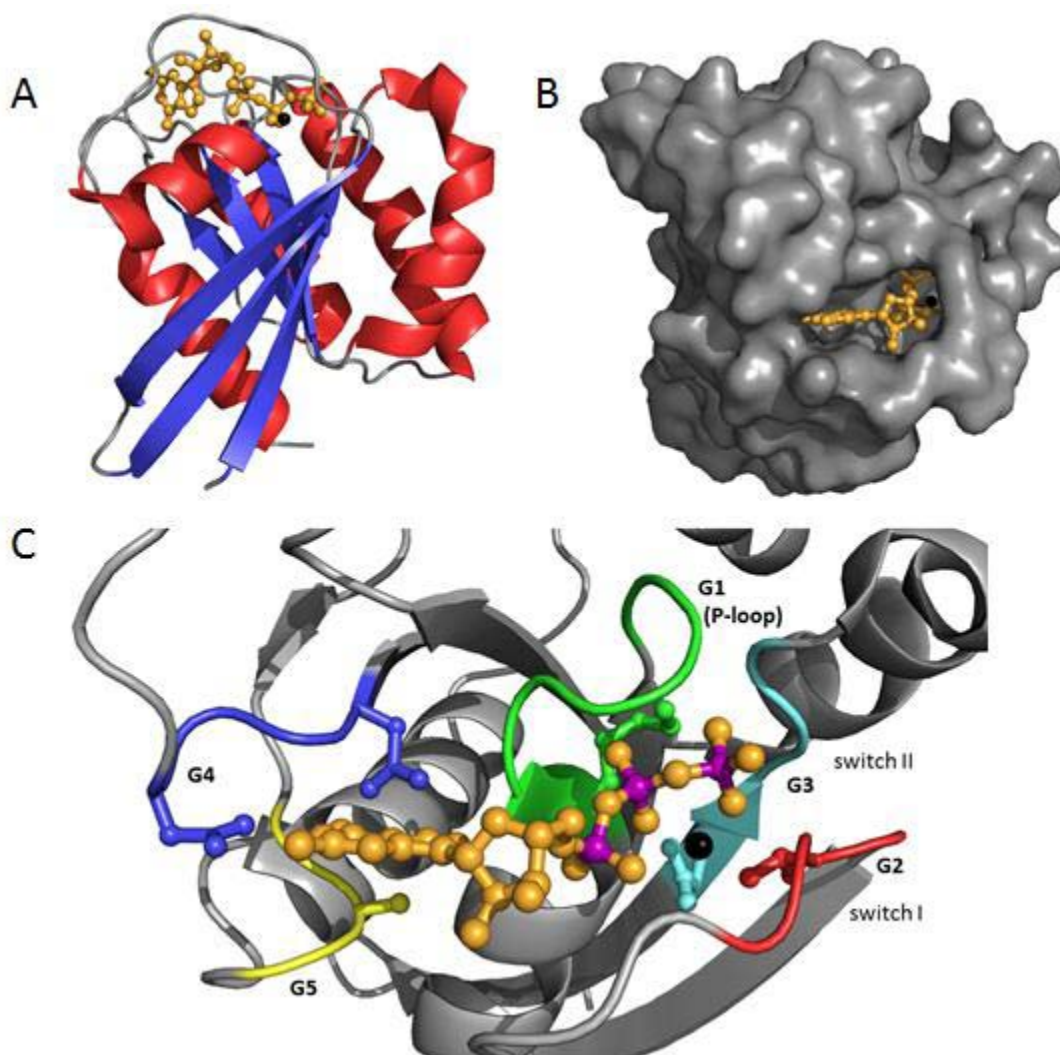


**Figure 1-10. GTP.** The three phosphate groups of GTP are denoted  $\alpha$ ,  $\beta$ , and  $\gamma$ . It is the  $\gamma$  phosphate which is hydrolyzed by GTPases.



### *The conserved G-domain*

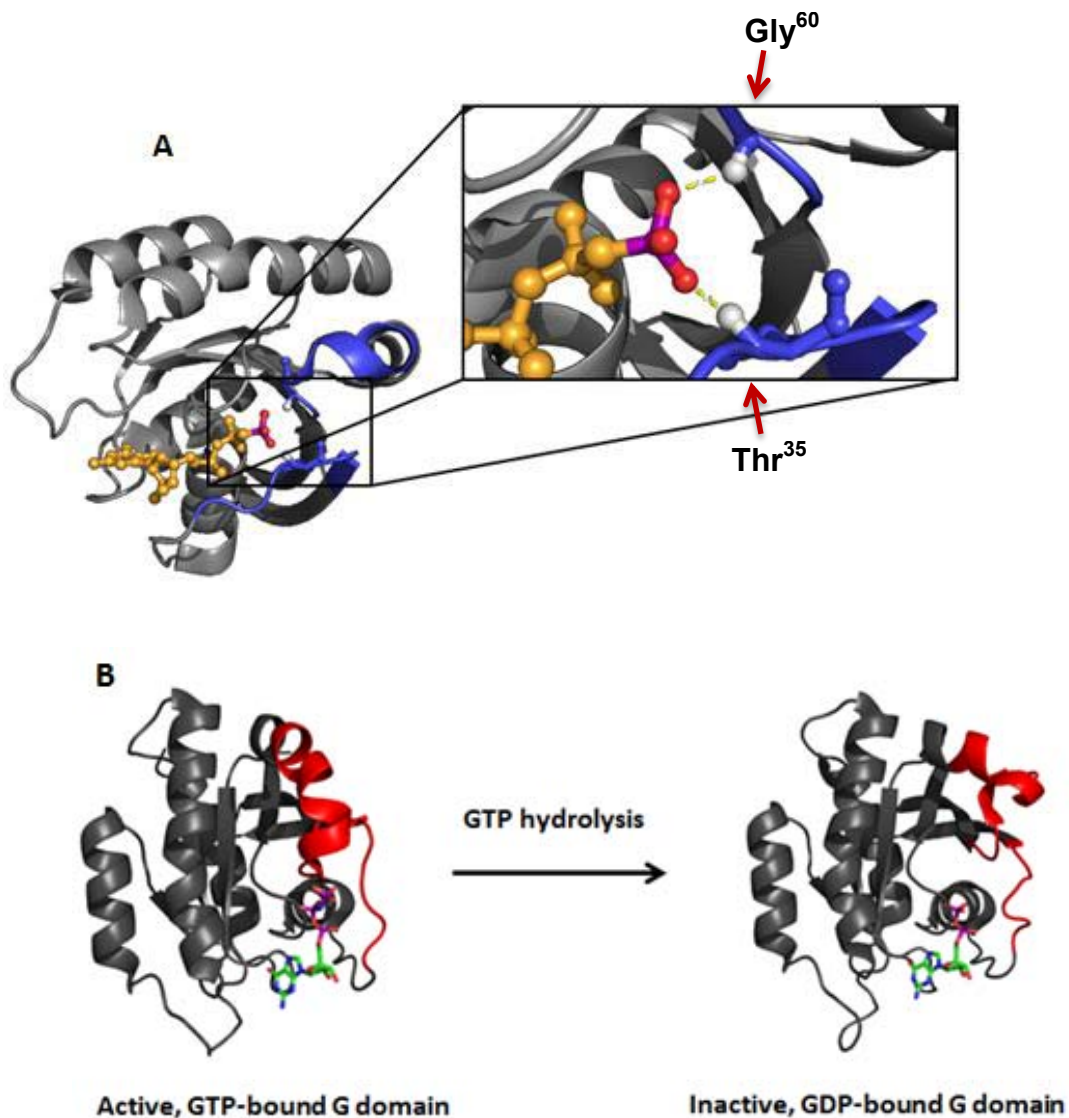
All GTPases share a common conserved domain called the G-domain, which is the key module involved in the binding and hydrolysis of GTP (Vetter, 2001). Figure 1-11 shows representations of the canonical G-domain, modeled from a high resolution crystal structure of the regulatory GTPase, Ras, bound to a non-hydrolyzable GTP analog, GDPNP (Buhrman et al., 2010). The GTP binding pocket is surrounded by a six-stranded  $\beta$ -sheet along with five  $\alpha$ -helices. There are five consensus sequences within the G domain which properly align a guanosine nucleotide in the binding site. The G1 consensus sequence GXXXXGKS/T (where X represents a variant residue), commonly referred to as the P-loop, is involved in the recognition of the  $\beta$  and  $\gamma$  phosphates of GTP (Saraste et al., 1990). The high affinity of guanine nucleotide binding is predominantly due to interactions between the phosphates, P loop, and a sequestered  $Mg^{2+}$  ion. The G2 (XXTX) and G3 (DXXG) consensus sequences also interact with the  $\beta$  and/or  $\gamma$  phosphates of GTP. Consensus sequences G4 (NKSD), and G5 (SAK) all contribute to the specific recognition of the guanine moiety of GTP/GDP (Vetter, 2001). Figure 1-11C also indicates the “switch I” and “switch II” regions of the G-domain, which are crucial for the conserved mechanism of GTPase function.



**Figure 1-11. The highly conserved G-domain.** (A) Ribbon structure of the Ras G-domain showing its characteristic  $\beta$ -sheet core surrounded by  $\alpha$ -helices. The GTP analog GDPNP is shown in orange. (B) Surface representation of the Ras G domain, showing the GTP binding pocket. (C) Close-up view of the conserved guanine nucleotide binding site, showing important interactions between conserved amino acid residues and the bound guanine nucleotide. PDBid 3K8Y.

### *GTPases – conformational change through molecular switching*

The highly conserved structural elements in GTPases are coupled with a common mechanism of action: conformational change induced by molecular switching. This molecular switching effect is the result of the hydrolysis of the terminal  $\gamma$ -phosphate of GTP, which causes a cascade of conformational rearrangements which are then transmitted through the GTPase. The canonical GTPase is considered “activated” when it is in the GTP-bound form. In this conformation (Figure 1-12A), two important hydrogen bonds are formed between the  $\gamma$ -phosphate group of GTP and backbone NH groups of two invariant residues: Thr<sup>35</sup> and Gly<sup>60</sup> (found within the switch I and switch II regions, respectively). This arrangement has been referred to as a “loaded spring” with the  $\gamma$ -phosphate acting as the hook (Vetter, 2001). Upon hydrolysis of GTP to produce GDP, the spring is released, allowing the switch I and II regions to relax into their GDP-bound “inactive” conformation (Figure 1-12B). The degree of conformational change upon GTP hydrolysis can differ significantly between GTPases. Many GTPases, such as the GTPase translation factors, have extensive peripheral structural elements to which these conformational changes are transmitted. For example, in the case of translation factor IF2, sequential conformational rearrangements act to transmit a structural change across a distance of 90 Å, from the G domain to the end of the protein (Roll-Mecak et al., 2000). Upon hydrolysis of GTP, the GTPase becomes “inactive” and GDP is jettisoned from the protein, to return to an empty, “waiting” state.



**Figure 1-12. Conformational changes of the G-domain upon hydrolysis of GTP.** (A) In the GTP-bound state, hydrogen bonds between two oxygens from the  $\gamma$ -phosphate of GTP and the amide hydrogens from two universally conserved residues, Thr<sup>35</sup> and Gly<sup>60</sup>, hold the switch I and II regions in a "tense" conformation. (B) Upon GTP hydrolysis, the switch I and II regions (red) undergo dramatic conformational changes, relaxing into their inactive, GDP-bound conformation.

*GTPase regulation: Guanine exchange factors (GEFs) & GTPase activating proteins (GAPs)*

Although GTPases exhibit regulatory functions in a variety of biological processes, they are themselves regulated by two additional types of biomolecules: guanine exchange factors (GEFs) and GTPase activating proteins (GAPs). Figure 1-13 illustrates the GTPase cycle. GEFs accelerate the rate of dissociation of GDP from GTPases (Rossman et al., 2005). Up to a  $10^5$ -fold rate enhancement has been observed for GEFs of a variety of well-studied GTPases (Zavialov et al., 2005). In the process of translation, the precise mechanism of guanine nucleotide exchange differs between the translational GTPases. For EF-Tu, a protein called EF-Ts acts as the GEF (Wieden et al., 2002). However, the ribosome acts as the GEF for RF3 and possibly for IF2 (Zavialov et al., 2001). For EF-G, it is debated whether the ribosome acts as the GEF (Zavialov et al., 2005) or if GDP spontaneously dissociates from the factor (Bourne et al., 1991). The GEF for LepA is unknown, but due to its strong homology with EF-G (Qin et al., 2006), it likely follows a similar mechanism to that of EF-G.

GAPs function by dramatically accelerating the GTP hydrolysis reaction, as the reaction is much too slow in the presence of GTPase alone to be of biological significance (Scheffzek et al., 1998). Essentially, GAPs are the molecular components responsible for rapidly switching GTPases from the “on” to “off” state. In the case of GTPase activation, an active debate has persisted

regarding the mechanism of activation. Unlike other aspects of GTPase function which show relatively strong evolutionary homology, activation by GAPs seems to follow a wider variety of mechanisms.

Investigations of the well-studied GTPase Ras have provided one widely cited mechanism of GTPase activation. For Ras, the GTPase reaction is catalyzed by the protein GAP-334 (Scheffzek, 1997). A crystal structure of the complex between Ras, GAP-334, and a ligand which mimics the transition state in the GTPase reaction ( $\text{GDP-AlF}_3$ ) suggests a crucial catalytic role for a specific arginine residue which is highly conserved among many known GAPs (Scheffzek, 1997). In this proposed “arginine finger” type mechanism, the positively charged guanidinium group of the arginine residue reaches into the active site, neutralizing developing charges and stabilizing the transition state (Scheffzek, 1997). This mechanism has been extended to several other known GAPs which share this conserved arginine finger motif (Vetter, 2001). However, there are many other examples of GAPs which do not appear to operate under the control of this arginine finger mechanism, and it has been postulated that many other mechanisms of GTPase activation by GAPs, independent of arginine, are likely to exist (Savelsbergh et al., 2000). In the case of GTPase translation factors, elements within the ribosome itself carry out the role GTPase activation (Brot et al., 1974; Parlato et al., 1981; Qin et al., 2006), which will be explored further in Chapter 2.

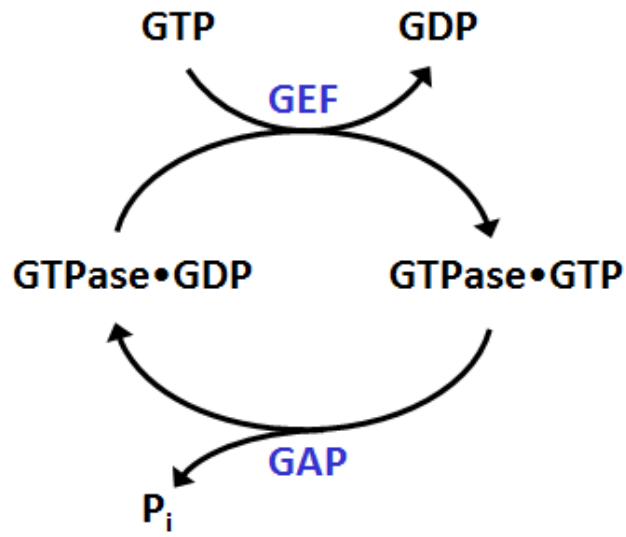
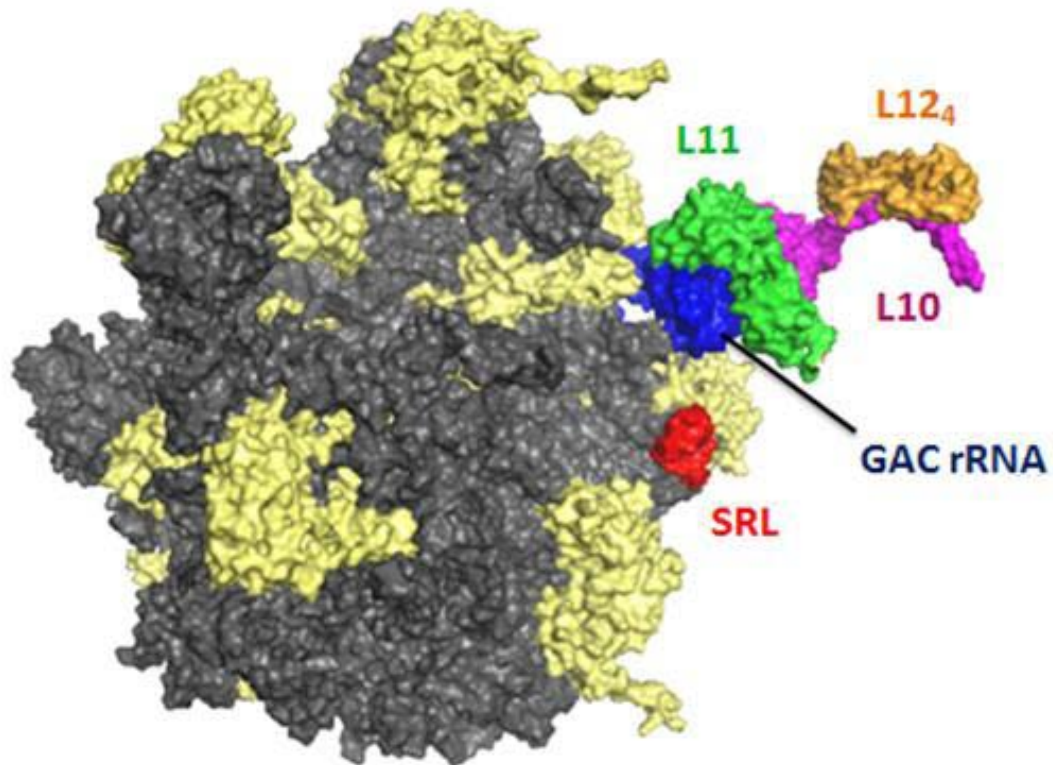


Figure 1-13. The GTPase cycle. GAPs stimulate GTP hydrolysis and GEFs promote the exchange of GTP for GDP.

### The translational GTPase binding region on the ribosome

Much research has been directed toward characterizing the interactions between translation factors and the ribosome. Noting that each of the known GTPase translation factors harbors the conserved G-domain and requires the presence of the ribosome to hydrolyze GTP and undergo guanine nucleotide exchange, early investigators hypothesized that there must be some conserved elements within the ribosome which interact with all GTPases. Indeed, through biochemical and structural experiments it has been determined that the GTPase translation factors IF-2, EF-Tu, EF-G, LepA, and RF3 interact at overlapping regions on the ribosome (Connell et al., 2008; Gao et al., 2007; La Teana et al., 2001; Moazed et al., 1988). This conserved GTPase binding site includes a region of 23S rRNA

called the sarcin-ricin loop (SRL) and a complex of 23S rRNA and ribosomal proteins collectively referred to as the GTPase-associated center (GAC). The ribosomal components which make molecular contacts with the translational GTPases are illustrated in Figure 1-14 and are discussed in further detail below.



**Figure 1-14. Regions of the ribosome which interact with the translational GTPases. Coloring of non-interacting regions: grey, rRNA; yellow, ribosomal proteins. Image created using a crystal structure of the 50S ribosomal subunit (PDBid 2WRJ)**



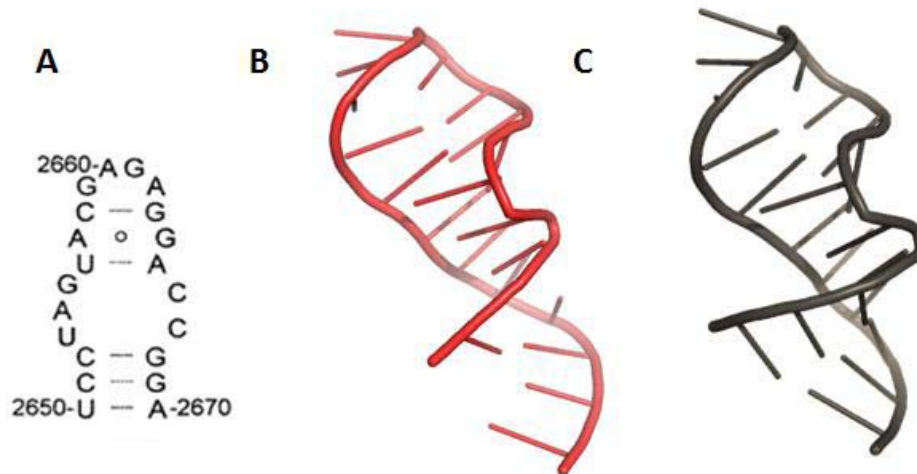
### *Sarcin-ricin loop*

The SRL rRNA (Figure 1-15), within helix 95 of 23S rRNA, contains a nearly universally conserved sequence of 12 rRNA nucleotides (bases 2654-2665) (Gutell et al., 1992). This region of rRNA is the target of the naturally occurring cytotoxins sarcin and ricin. Sarcin, a ribonuclease of fungal origin, hydrolyzes the phosphodiester bond on the 3' side of G2661 (Huber and Wool, 1988). Ricin, an N-glycosidase from castor beans (*Ricinus communis*), depurinates A2660 via hydrolysis of its N-glycosidic bond (Huber and Wool, 1988). Although these toxins only catalyze these single covalent modifications, complete inactivation of the ribosome results from interaction with either sarcin or ricin. The dramatic effects of these toxins provided an early indication that the SRL is essential for ribosome function (Huber and Wool, 1988).

X-ray crystal structures show that tertiary contacts link the SRL with many other important regions of the 50S subunit (Ban et al., 2000). The SRL makes loop-loop interactions with helix 91 of 23S rRNA, which in turn interacts with helices 89, 90, and 92, regions that are coupled directly to the A and P sites and the peptidyl transferase center (PTC) (Lancaster et al., 2008). These interactions suggest the existence of a signaling pathway between the SRL and the functional core of the ribosome which can be activated via GTPase factor binding.

Invoking a clever reductionist approach to studying the interaction between the SRL and translation factors, it has been observed that in vitro transcribed RNA

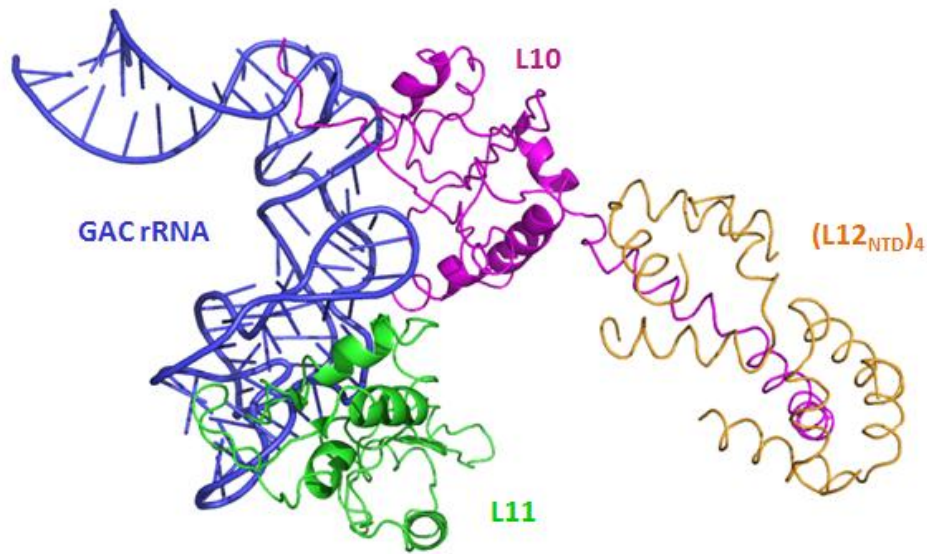
fragments which mimic the SRL retain the capability to bind EF-G and EF-Tu with affinities that are within an order of magnitude of those for the binding of these factors to intact ribosomes (Munishkin and Wool, 1997). In addition, high resolution crystal structures of these rRNA mimics and RNA-factor complexes have revealed that the in vitro transcribed SRL fragments retain wild-type structure (Figure 1-15) (Correll et al., 1998).



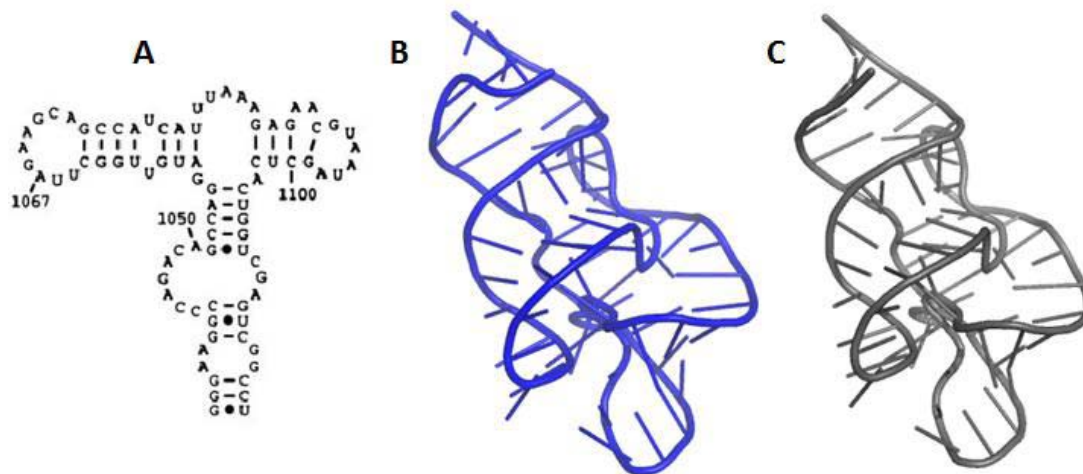
**Figure 1-15. The sarcin-ricin loop of 23S rRNA. (A) Secondary structure diagram of the SRL. (B) Tertiary structure of the SRL from a crystal structure of the 70S ribosome (PBDId 2WRJ). (C) Crystal structure of an in vitro transcribed SRL mimic (PBDId 430D). Comparison of (B) and (C) clearly reveals that the SRL mimic folds into a tertiary structure which resembles its native conformation.**

### *GTPase associated center*

The GAC (Figure 1-16) is comprised of a highly conserved region of 23S rRNA and three ribosomal proteins, L10, L11, and L7/12 (L7 is identical to L12, except for its acetylated N terminus; the two proteins will hereafter be collectively referred to as L12). In *E. coli*, L10 and L12 form a pentameric complex, L10(L12)<sub>4</sub>, in which two L12 dimers bind to the extended  $\alpha$ -helical C-terminus of L10; however, in thermophilic bacteria, three L12 dimers associate with L10 to form the heptamer L10(L12)<sub>6</sub> (Diaconu et al., 2005). Along with ribosomal protein L11, this complex binds to a conserved region of 23S rRNA (GAC rRNA) within nucleotides 1030-1124 (Figures 1-14 and 1-16) (Beauclerk et al., 1984). As with the SRL, in vitro synthesized GAC rRNA mimics have been shown to fold into a native-like tertiary structure (Figure 1-17) and retain the ability to bind L10, L11, and elongation factor G (Diaconu et al., 2005; Munishkin and Wool, 1997; Wimberly et al., 1999). The entire GAC complex, commonly referred to as the ribosomal “stalk” due to its appearance in cryoEM images as a lateral protuberance extending from the surface of the ribosome, has been shown to be a highly flexible and functionally important component of the ribosome (Beauclerk et al., 1984; Diaconu et al., 2005). There has been intensive interest in elucidating the role of ribosomal protein L12 in various ribosome functions.



**Figure 1-16.** Components of the GTPase associated center, from a crystal structure of the 70S ribosome (PDBid 2WRJ). Note that in crystal structures of the ribosome, the dynamic CTD of L12 is not typically resolved.



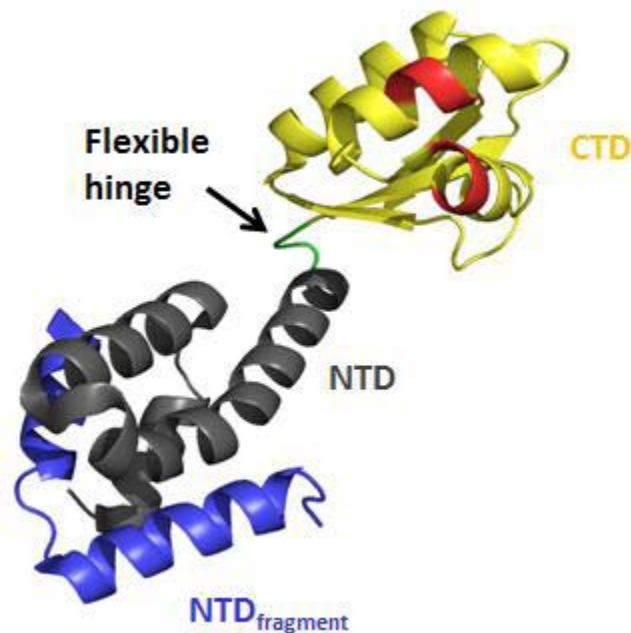
**Figure 1-17.** GAC rRNA. (A) Secondary structure diagram. (B) Tertiary structure of the GAC rRNA from a crystal structure of the 70S ribosome (PDBid 2WRJ). (C) Crystal structure of an in vitro transcribed GAC rRNA mimic (PDBid 430D). Comparison of (B) and (C) reveals a remarkable degree of similarity between the GAC rRNA mimic and the same region of rRNA within the context of the ribosome.

### *Ribosomal protein L12*

The 12 kDa ribosomal protein L12 (Figure 1-18) consists of an N-terminal domain, responsible for dimerization, which is connected via a flexible hinge region to a globular C-terminal domain (CTD) (Diaconu et al., 2005; Liljas and Gudkov, 1987; Moller and Castleman, 1967). It is notable for being the only ribosomal protein present in multiple copies (two dimers) and for not making any direct molecular contacts with rRNA – it associates with the ribosome exclusively through interactions with the extended alpha helical CTD of protein L10, which itself is bound to GAC rRNA via its N-terminal domain (Iben and Draper, 2008). Because of its unique properties, L12 has been the focus of numerous investigations (Datta et al., 2005; Diaconu et al., 2005; Savelsbergh et al., 2000).

Many studies have attempted to elucidate the function of L12 in the translational cycle, although the results and implications of these experiments have not been sufficiently reconciled into a unified explanation of L12 function. There is competing evidence that L12 plays a role in factor binding, GTP hydrolysis (discussed further in Chapter 2), and release of inorganic phosphate, although its relative contributions to each process are actively debated (Diaconu et al., 2005; Savelsbergh et al., 2005). A recent NMR study confirmed that a conserved region of the L12 CTD interacts with all of the translational GTPases (this region is shown in red in Figure 1-18), although LepA was not included in the experiments (Helgstrand et al., 2007). Interestingly, a report by Uchiumi et al

found that substitution of L12 on *E. coli* ribosomes with the eukaryotic homolog, P1/P2, resulted in hybrid ribosomes that recognized only eukaryotic elongation factor 2 and did not bind to prokaryotic EF-G (Uchiumi et al., 2002). This is a remarkable result, which suggests that L12 may play an active role in factor discrimination.



**Figure 1-18.** Crystal structure of full-length ribosomal protein L12 bound to a L12-NTD fragment. L12 consists of an  $\alpha$ -helical NTD (grey, shown interacting with an additional NTD fragment indicated in blue), responsible for dimerization and association with the ribosome (see Figure 15), and a globular CTD (yellow), which interacts with translation factors. These two domains are connected via a flexible hinge region (green). Conserved regions of the CTD which have been shown by NMR to interact with translation factors are colored red.

## **Chapter 2 – Activation of GTPase translation factors on the ribosome**

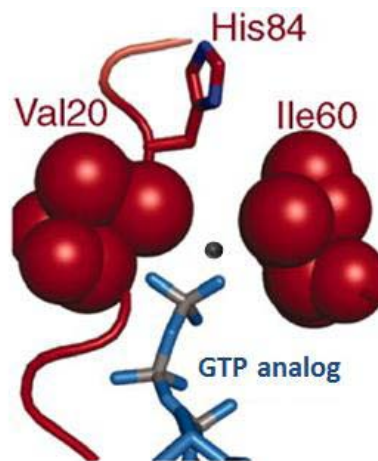
### **Introduction**

#### *GTPase activation of translation factors*

In accordance with the broad paradigm of GTPase functionality, the intrinsic GTP hydrolysis activity of the translational GTPases is negligible (Parmeggiani and Sander, 1981). As mentioned in Chapter 1, the activity of these factors is strongly enhanced in the presence of the ribosome (Parlato et al., 1981; Rodnina et al., 1995). However, the identity of the ribosomal component responsible for this GTPase activation is unknown, despite numerous efforts directed at its elucidation (Mohr et al., 2002).

Structural and biochemical studies of the translational GTPases have revealed that several conserved residues within the G domain of these factors are essential for ribosome-mediated GTP hydrolysis. Crucial to this reaction is an invariant histidine (His<sup>84</sup> in the EF-Tu numbering scheme) found within the switch II region of the translational GTPases. Replacement of His<sup>84</sup> with Ala in *E. coli* EF-Tu decreases the rate of ribosome-dependent GTP hydrolysis by EF-Tu greater than 10<sup>6</sup>-fold, but the earlier steps of EF-Tu•GTP•aminoacyl-tRNA ternary complex binding to the ribosome and codon recognition are only marginally affected (Daviter et al., 2003). Structural evidence suggests that His<sup>84</sup> acts as a

general base, abstracting a proton from a water molecule and coordinating it for a nucleophilic attack on the  $\gamma$ -phosphate of GTP (Eccleston and Webb, 1982; Kjeldgaard et al., 1993; Voorhees et al., 2010). This interaction is thought to be regulated by a “hydrophobic gate” comprised of Val<sup>20</sup> and Ile<sup>60</sup>, residues which are proposed to restrict the ability of His<sup>84</sup> to interact with the catalytic water (Figure 2-1) (Berchtold et al., 1993; Voorhees et al., 2010). Activation of the translational GTPases via interaction with ribosomal components is proposed to “open” this gate, allowing His<sup>84</sup> to carry out its catalytic role (Berchtold et al., 1993). Components of the ribosome which could accomplish this task include the region comprising the GTPase factor binding site (discussed in Chapter 1), including the SRL or GAC rRNA, or ribosomal proteins L10, L11, or L12 (Figure 1-13).



**Figure 2-1.** The GTPase active site of EF-Tu, from a x-ray crystal structure of EF-Tu bound to a non-hydrolyzable GTP analog, indicating the essential residue His<sup>84</sup>. Also shown are residues Val<sup>20</sup> and Ile<sup>60</sup>, which comprise the regulatory “hydrophobic gate” that occludes the catalytic water (grey sphere) prior to GTPase activation. Figure adapted from Voorhees, et al. (2010), based on a x-ray crystal structure determined by Kjeldgaard, et al. (1993).



### *Role of the SRL/GAC in GTPase activation*

A still unresolved and much debated question is whether the regions of the ribosome which make contacts with the translational GTPases, the SRL and GAC (described in Chapter 1), serve simply as static factor-binding sites, or if the interaction between one or both these regions and translation factors triggers conformational changes that allow GTP hydrolysis to occur (Lancaster et al., 2008).

A current candidate for the ribosomal component which is responsible for GTPase activation is ribosomal protein L12. Experiments involving the extraction of L12 from ribosomes have shown that the presence of L12 is required for ribosome-dependent GTP hydrolysis by translation factors (Brot et al., 1974; Donner et al., 1978; Savelsbergh et al., 2005). In 2000, Savelsbergh et al. published a study in which they investigated the interaction between free L12 and EF-G in the absence of ribosomes (Savelsbergh et al., 2000). Their results indicated that free L12 strongly stimulates GTP hydrolysis by EF-G, suggesting that L12 may be the ribosomal component which activates the GTPase translation factors (i.e. the GAP) (Savelsbergh et al., 2000). This study has been cited as evidence of the role of L12 as ribosomal GAP (Diaconu et al., 2005; Mohr et al., 2002). However, it was noted by Savelsbergh et al. that the rate of GTP hydrolysis by EF-G in the presence of L12 is much slower than in the presence of active ribosomes, suggesting that L12 is either not the sole element

involved in factor GTPase stimulation or its conformation free in solution is not properly suited to efficiently stimulate GTP hydrolysis (Savelsbergh et al., 2000).

A recent publication by Clementi et al implicated a specific region of the SRL rRNA as essential for GTP hydrolysis by EF-G (Clementi et al., 2010). By applying a novel “atomic mutagenesis” approach, which allowed the systematic alteration of specific functional groups on 23S rRNA nucleotides, the experiments of Clementi et al suggest that the purine base of the universal nucleotide A2660 may be involved in triggering GTP hydrolysis of EF-G (Clementi et al., 2010). However, further experiments are necessary to test the validity of this novel technique and to evaluate whether the SRL does indeed participate in translational GTPase activation.

## **Research Aims**

The identity of the ribosomal component responsible for GTPase activation is unknown. The goal of this work is to further clarify the roles of the factor-binding regions of the ribosome, the SRL and GAC, in GTPase activation. Emphasis will be placed on the interactions between GTPases and ribosomal protein L12. Since no previously published studies of this nature have examined all GTPase translation factors together, attempts will be made to find common patterns of activation and/or inhibition which can help formulate a generalized view of ribosome-GTPase interactions. Also, each of the individual components comprising the ribosomal binding site of the translational GTPases will be purified and tested for their ability to stimulate GTP hydrolysis activity.

## Materials and Methods

### *Buffers*

GTPase lysis buffer: 50 mM Tris-HCl (pH 7.5), 60 mM NH<sub>4</sub>Cl, 7 mM MgCl<sub>2</sub>, 15 mM imidazole, 25 % glycerol, 6 mM β-mercaptoethanol.

GTPase wash buffer: 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 60 mM NH<sub>4</sub>Cl, 7 mM MgCl<sub>2</sub>, 15 mM imidazole, 25 % glycerol, 6 mM β-mercaptoethanol.

GTPase elution buffer: 50 mM Tris-HCl (pH 7.5), 60 mM NH<sub>4</sub>Cl, 7 mM MgCl<sub>2</sub>, 250 mM imidazole, 25 % glycerol, 6 mM β-mercaptoethanol.

GTPase storage buffer: 50 mM Tris-HCl (pH 7.5), 60 mM NH<sub>4</sub>Cl, 7 mM MgCl<sub>2</sub>, 50 % glycerol, 1 mM dithiothreitol (DTT).

JE28 lysis buffer: 20 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 150 mM KCl, 30 mM NH<sub>4</sub>Cl, 5 mM imidazole, 1 mM DTT.

JE28 wash buffer: 20 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 150 mM KCl, 500 mM NH<sub>4</sub>Cl, 5 mM imidazole, 1 mM DTT.

JE28 SW buffer: 20 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 150 mM KCl, 500 mM NH<sub>4</sub>Cl, 1 mM DTT.

JE28 elution buffer: 20 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 150 mM KCl, 30 mM NH<sub>4</sub>Cl, 150 mM imidazole, 1 mM DTT

Ribosome storage buffer: 50 mM Tris-HCl (pH 7.5), 30 mM NH<sub>4</sub>Cl, 7 mM MgCl<sub>2</sub>,  
25 % glycerol

L12 extraction buffer: 20 mM Tris-HCl (pH 7.5), 1.0 M NH<sub>4</sub>Cl, 20 mM MgCl<sub>2</sub>, 50  
% glycerol, 5 mM β-mercaptoethanol.

RNA transcription buffer: 40 mM Tris-HCl (pH 7.5), 1 mM spermidine, 10 mM  
MgCl<sub>2</sub>, 30 mM NH<sub>4</sub>Cl, 5 mM DTT.

GTPase reaction buffer: 90 mM K-HEPES (pH 7.5), 100 mM NH<sub>4</sub>Cl, 20 mM  
Mg(OAc)<sub>2</sub>.

#### *Construction of (His)<sub>6</sub>-tagged translation factor overexpression clones*

Entire genes encoding translation factors IF2, EF-G, LepA, and RF3, were PCR amplified from the genomic DNA of *E. coli* with primers that introduced upstream BamHI and downstream XhoI restriction sites, as well as an N-terminal (His)<sub>6</sub> tag. Gene constructs were cloned into the pSV expression vector. Sequences of the expression vectors were verified by Nevada Genomics

#### *Overexpression of (His)<sub>6</sub>-tagged translation factors*

10 mL cultures of *E. coli* BL21(DE3) cells transformed with the pSV constructs were grown overnight at 37° C in Lysogeny broth (LB, 10% w/v BactoTryptone,

5% w/v yeast extract, 10% w/v sodium chloride), in the presence of 35 µg/ml kanamycin. 1 L cultures of LB/kanamycin were inoculated with the overnight cultures and then shaken at 37° C until the optical density at 600 nm reached a value of 0.5 (mid-log phase). Cultures were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), the temperature was adjusted to 15° C, and cell cultures were allowed to shake overnight. Cells were harvested by centrifugation (GS3 rotor, 4° C, 6000 rpm, 15 minutes). Pelleted cells from 2 L of cell culture were resuspended in 35 mL GTPase lysis buffer in the presence of 1 mg/mL lysozyme and 1 mM phenylmethanesulfonyl fluoride (PMSF). After incubation of the resuspended cells on ice for 30 minutes, cells were lysed by sonication (50% duty cycle, 3-5 minutes). The lysate was clarified by centrifugation (SS34 rotor, 4° C, 18000 rpm, 30 minutes) and filtered through a 0.45 µm syringe filter before being subjected to affinity chromatography.

#### *Purification of (His)<sub>6</sub>-tagged translation factors and ribosomal proteins*

For affinity purification, a column was packed with 10 mL of Ni<sup>2+</sup>• nitrilotriacetic acid (Ni-NTA) resin (Quiagen), connected to a BioCAD FPLC system, and equilibrated with GTPase lysis buffer. After loading the lysate, the column was washed with GTPase lysis buffer until the A<sub>280</sub> absorbance reached baseline. The resin was then washed with at least two column volumes of GTPase wash buffer, and one additional volume of GTPase lysis buffer. The (His)<sub>6</sub>-tagged

translation factors were then eluted with a linear imidazole gradient. 1 mL fractions were collected on ice throughout the elution gradient. Fractions judged from SDS-PAGE to contain the desired protein were then pooled and dialyzed overnight in 1 L GTPase storage buffer at 4° C. After quantification based on the absorbance of 280 nm wavelength UV radiation ( $A_{280}$ ) or the Bradford assay (Kruger, 1994), proteins were divided into 1.0 mL aliquots, flash-frozen in liquid nitrogen, and stored at -80° C until further use.

Affinity tagged ribosomal proteins L10, L11, and L12 were purified in the unfolded state (to prevent co-purification of bound ribosomes), by including 7 M urea in the affinity purification buffers. Additionally, pooled fractions containing purified, denatured ribosomal proteins were subjected to ultracentrifugation at 150,000 x g for 2 hours (60Ti rotor, 57400 rpm, 4°C). Finally, the ribosomal proteins were refolded by dialyzing twice into 1 L of storage buffer lacking urea, quantified using the Bradford assay, flash-frozen in liquid nitrogen, and stored at -80° C until further use.

#### *Growth and purification of tetra-(His)<sub>6</sub>-tagged ribosomes from E. coli JE28*

*E. coli* JE28 cell freezer stocks were used to prepare 10 mL overnight cultures in LB supplemented with 35 µg/ml kanamycin. After growing overnight at 37° C, the overnight cultures were diluted 1:10 into 1 L of LB with 35 µg/ml kanamycin and

shaken at 37° C. At  $A_{600} = 1.0$ , the cultures were placed on ice and cooled to 4° C, with occasional manual shaking to ensure even heat distribution. Cells here harvested and lysed as described above, with the exception of using JE28 lysis buffer instead. Clarified, filtered lysate was loaded onto the Ni-NTA resin, which was washed with JE28 lysis buffer until  $A_{280}$  reached baseline, and then extensively washed (at least four column volumes) with JE28 wash buffer. Ribosomes were eluted with JE28 elution buffer, pooled immediately, and dialyzed overnight in JE28 dialysis buffer. The ribosomes were then pelleted via ultracentrifugation at 150,000 x g (60Ti rotor, 57400 rpm, 2 hr, 4° C), resuspended in JE28 SW buffer, pelleted again, and then resuspended in ribosome buffer. After quantification based on  $A_{260}/A_{280}$  measurements, ribosomes were divided into small aliquots, flash-frozen in liquid nitrogen, and stored at -80° C until further use.

### *Circular dichroism*

Circular dichroism (CD) spectra were performed on an Olis DSM 20 CD instrument. Proteins were diluted with GTPase storage buffer to a final concentration of 0.5 mg/mL and ellipticity was recorded from 200 to 270 nm at 20° C, using 1 nm steps.



### *Electrophoresis*

Discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described (Cleveland et al., 1977). The percentage (% w/v) of acrylamide used in gels is indicated in figure legends. Samples were diluted into a reducing load dye (2% w/v SDS, 80 mM Tris-HCl [pH 6.8], 10% v/v glycerol, 0.002% w/v bromophenol blue, 5% v/v  $\beta$ -mercaptoethanol) and were denatured by heating at 90° C for 3 minutes before loading on gels. The applied electric field was typically 120 V. Gels were stained using Coomassie brilliant blue dye and destained using a 50:40:10 H<sub>2</sub>O:methanol:glacial acetic acid solution. Molecular weight reference standards were purchased from Fisher BioReagents.

### *Depletion and reconstitution of L12 from 70S ribosomes*

L12 was removed from 70S ribosomes via treatment with NH<sub>4</sub>Cl/ethanol according to the method of Mohr et al., with modifications (Mohr et al., 2002). 450 pmol of purified 70S ribosomes were incubated in 450  $\mu$ L of L12 extraction buffer at 4° C for 5 minutes. 250  $\mu$ L of ice-cold absolute ethanol was added, and the mixture was stirred at 4° C. After 5 min, an additional 250  $\mu$ L of ethanol was added and the mixture was stirred for an additional 5 minutes. The mixture was then centrifuged at 150,000 x g for 30 minutes, resulting in a visible white pellet.

Immediately after centrifugation, the supernatant was removed by decanting and saved for SDS-PAGE analysis. Centrifuge tubes were inverted for 5 minutes to remove residual supernatant, and the pellet consisting of depleted ribosomes was resuspended in ribosome buffer by gentle pipetting. For reconstitution, depleted ribosomes were incubated with a 5-fold excess of purified (His)<sub>6</sub>-tagged L12 for 30 minutes at 37° C.

#### *Analysis of L12 extraction supernatant*

To assess the removal of proteins from depleted 70S ribosomes, proteins in the extraction supernatant were precipitated by addition of 5 volumes of acetone, precooled to -20° C. Samples were incubated at -20° C for 1 hour prior to centrifugation at 3500 rpm for 10 min. The supernatant was then decanted and residual acetone was removed by air-drying at room temperature. Precipitated protein was then resuspended in 100 µL of GTPase storage buffer and subjected to SDS-PAGE analysis.

#### *In vitro transcription of RNA fragments corresponding to SRL or GAC rRNA*

Oligoribonucleotide rRNA mimics were synthesized using T7 RNA polymerase and synthetic DNA templates (Milligan et al., 1987). The templates consisted of a double-stranded T7 promoter adjacent to a single-stranded region

complementary to the sequence to be transcribed. 500 pmol of each of the two DNA oligomers used for the template were diluted to a final volume of 500  $\mu$ L and annealed by heating in a 90° C water bath for 3 minutes, followed by slowly cooling to room temperature. The transcription reaction (1 mL) was carried out in RNA transcription buffer, and consisted of 500 nM DNA template, 1.5 mM of each of the four nucleoside triphosphates (ATP, TTP, GTP, and CTP), and 3  $\mu$ L of T7 RNA polymerase. After incubating at 37° C for 4 hours, sodium acetate (pH 5.2) was added to a final concentration of 400 mM and EDTA (pH 8.0) was added to a final concentration of 20 mM. The transcription reaction mixture was then extracted with phenol and chloroform and the nucleic acids were precipitated with absolute ethanol. Precipitated RNA was pelleted by centrifuging at 13,000 rpm for 20 minutes, the supernatant was removed by decanting, and residual ethanol was removed by centrifugation under vacuum in a SpeedVac. RNA pellets were dissolved in 20  $\mu$ L H<sub>2</sub>O and heated at 90° C for 1 minute prior to electrophoresis in 20% w/v polyacrylamide gels containing 7 M urea. Gels were typically run at a constant power of 15 W for 1.5 hours, at 4° C. Bands were visualized by UV shadowing, excised, and RNA was eluted by crushing the excised band and soaking it overnight in a solution containing 500 mM sodium acetate (pH 5.2) and 0.1 mM EDTA. Purified RNA was precipitated with ethanol and dissolved in RNA buffer. RNA oligonucleotides were renatured by heating at 90° C for 2 minutes followed by cooling at 4° C overnight. After quantification via

$A_{260}$  absorbance, samples were either immediately used for experiments or flash-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

### *Fluorescence*

Fluorescence emission spectra of EF-G•GDPNP and EF-G•GDPNP•RNA complexes were acquired on a PTI spectrofluorometer. All measurements were carried out at room temperature. 65  $\mu\text{L}$  samples were prepared in GTPase reaction buffer, and consisted of 10  $\mu\text{M}$  EF-G, 0.8 mM GDPNP, and/or 20  $\mu\text{M}$  RNA oligonucleotides, as indicated. Samples were incubated for 10 minutes at  $37^{\circ}\text{C}$ , followed by incubation at room temperature for 10 minutes. Samples were excited at 280 nm and the emission spectrum from 300-400 nm was recorded (excitation and emission slit widths were 1.5 mm).

### *GTP Hydrolysis*

GTP hydrolysis was measured using the method of Frolova et al, with modifications (Frolova et al., 1996). Reaction mixtures consisted of ribosomes, translation factors,  $[\gamma\text{-}^{32}\text{P}]\text{GTP}$  and/or ribosomal proteins, at concentrations indicated in figure legends. Reaction components (without GTP) were mixed with GTPase reaction buffer, incubated at  $37^{\circ}\text{C}$  for 20 minutes to facilitate complex formation, and the reaction was initiated by addition of  $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ . The

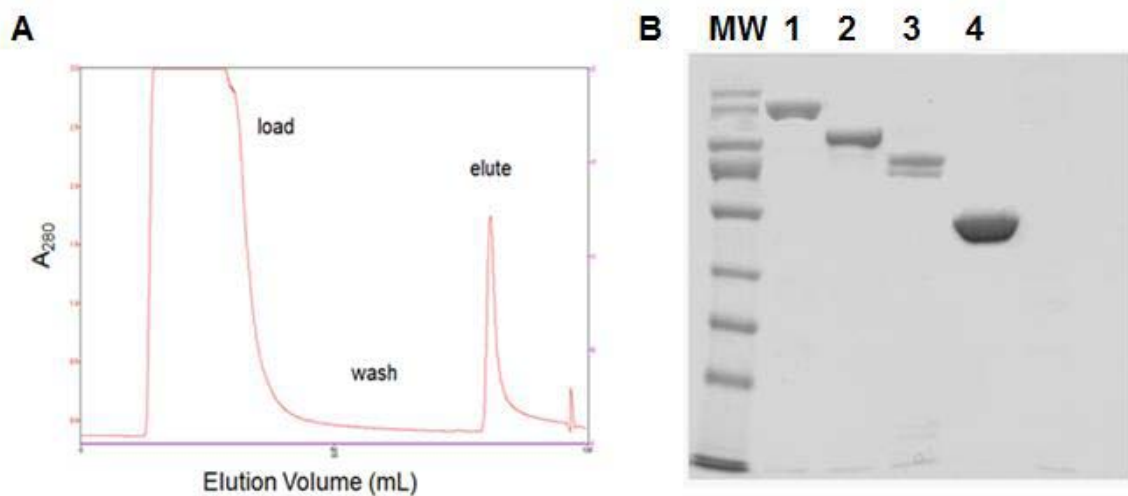
reaction was carried out at 37° C. To quench the reaction, 20 µL aliquots were removed at specified time points and thoroughly mixed with 380 µL of 5% w/v activated charcoal in 50 mM NaH<sub>2</sub>PO<sub>4</sub>, on ice. These mixtures were then centrifuged at 10,000 rpm for 10 min at 4° C and hydrolyzed <sup>32</sup>P<sub>i</sub> released into the supernatant was quantified by mixing 50 µL of supernatant with scintillation cocktail followed by liquid scintillation counting on a MicroBeta scintillation counter. Standard curves were generated by preparing serial dilutions of solutions containing a known quantity of [γ-<sup>32</sup>P]GTP and subjecting these standards to liquid scintillation counting.

## Results

### *Expression & purification of GTPases and ribosomal proteins*

A standard scheme was used for the cloning, expression, and purification of all proteins. Genes were cloned into the pSV expression vector from *E. coli* genomic DNA. This vector harbors a kanamycin resistance gene and also introduces a N-terminal (His)<sub>6</sub> tag adjacent to a TEV protease recognition sequence. *E. coli* cells transformed with these vectors were grown, induced, harvested, and lysed using standard techniques (Materials and Methods). All proteins were expressed in the soluble fraction, with the exception of ribosomal protein L10, which was found to be expressed in inclusion bodies and was resolubilized by treatment with urea. All proteins were purified via affinity chromatography on resin containing immobilized Ni<sup>2+</sup> ions, which bind with high affinity to the N-terminal (His)<sub>6</sub> tag. After extensively washing the resin to remove unwanted contaminants, proteins were eluted by applying an imidazole gradient. Figure 2-2 shows a typical elution profile which follows the standard purification scheme. Translation factors were purified in native conditions, resulting in high yields, as assessed by measuring the absorbance of UV light at 280 nm and using a calculated estimation of the molar extinction coefficient to determine the concentrations via the Beer-Lambert Law. Figure 2-2 shows an SDS-PAGE gel of all purified GTPases, which indicates that the one-step affinity purification results in highly purified protein with minimal contaminants. All proteins migrate

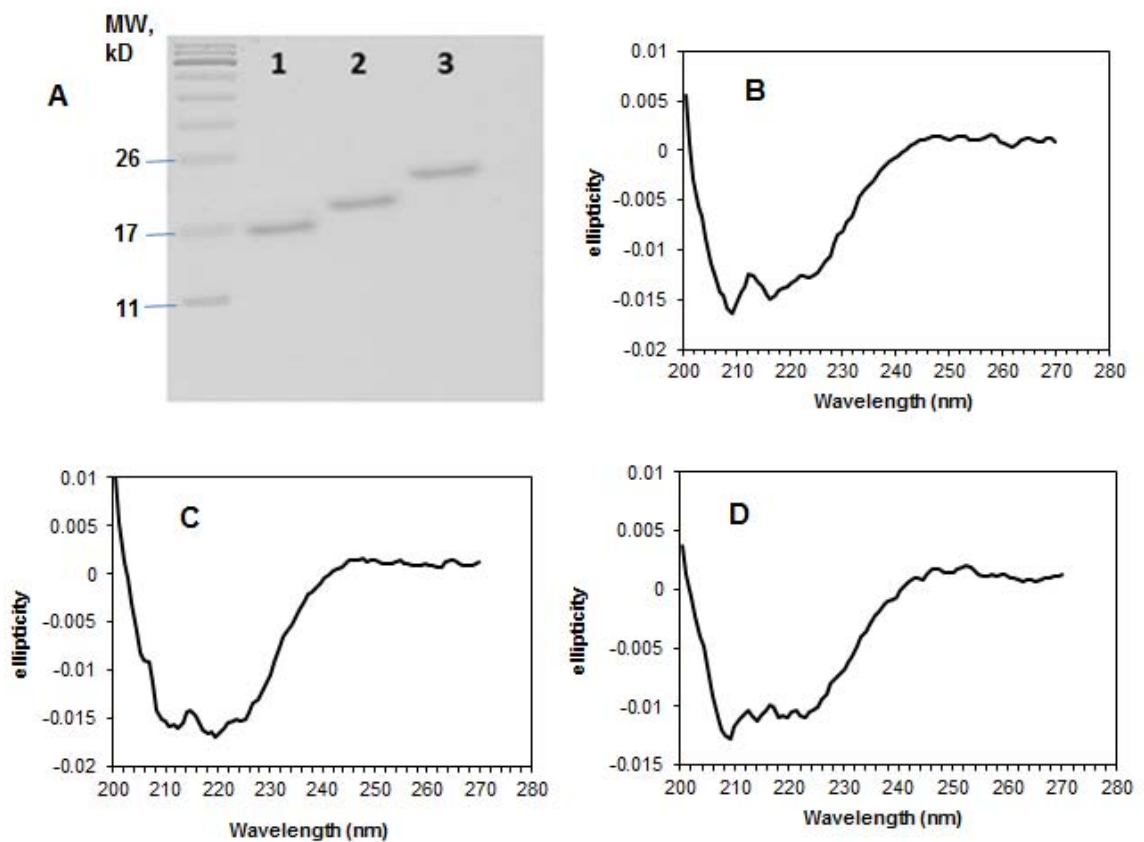
as a single band, with the exception of LepA, which contains two closely spaced bands. The identity of the lower molecular weight band in the LepA sample may be due to cleavage of the novel CTD of LepA, but this has not yet been confirmed.



**Figure 2-2. A typical FPLC elution profile for the general purification of GTPases (left) and a 10 % SDS-PAGE gel showing all purified GTPases (right); lanes: (MW) Molecular weight standards, (1) IF2, (2) EF-G, (3) LepA, (4) RF3**

Purification of ribosomal proteins required additional steps to avoid contamination by bound endogenous ribosomes. To achieve this, ribosomal proteins were purified under denaturing conditions and pooled fractions were subjected to ultracentrifugation, followed by refolding of the proteins under native buffer conditions. SDS-PAGE analysis of the stalk proteins (Figure 2-3A) reveals a

high level of electrophoretic purity, with all proteins migrating as a single band. Figure 2-3,B-D shows circular dichroism spectra of ribosomal proteins L10, L11, and L12, which all bear the characteristic hallmarks of folded  $\alpha$ -helical proteins, which is expected. As L10, L11 and L12 do not contain any tryptophan residues, they could only be quantified with the Bradford assay.

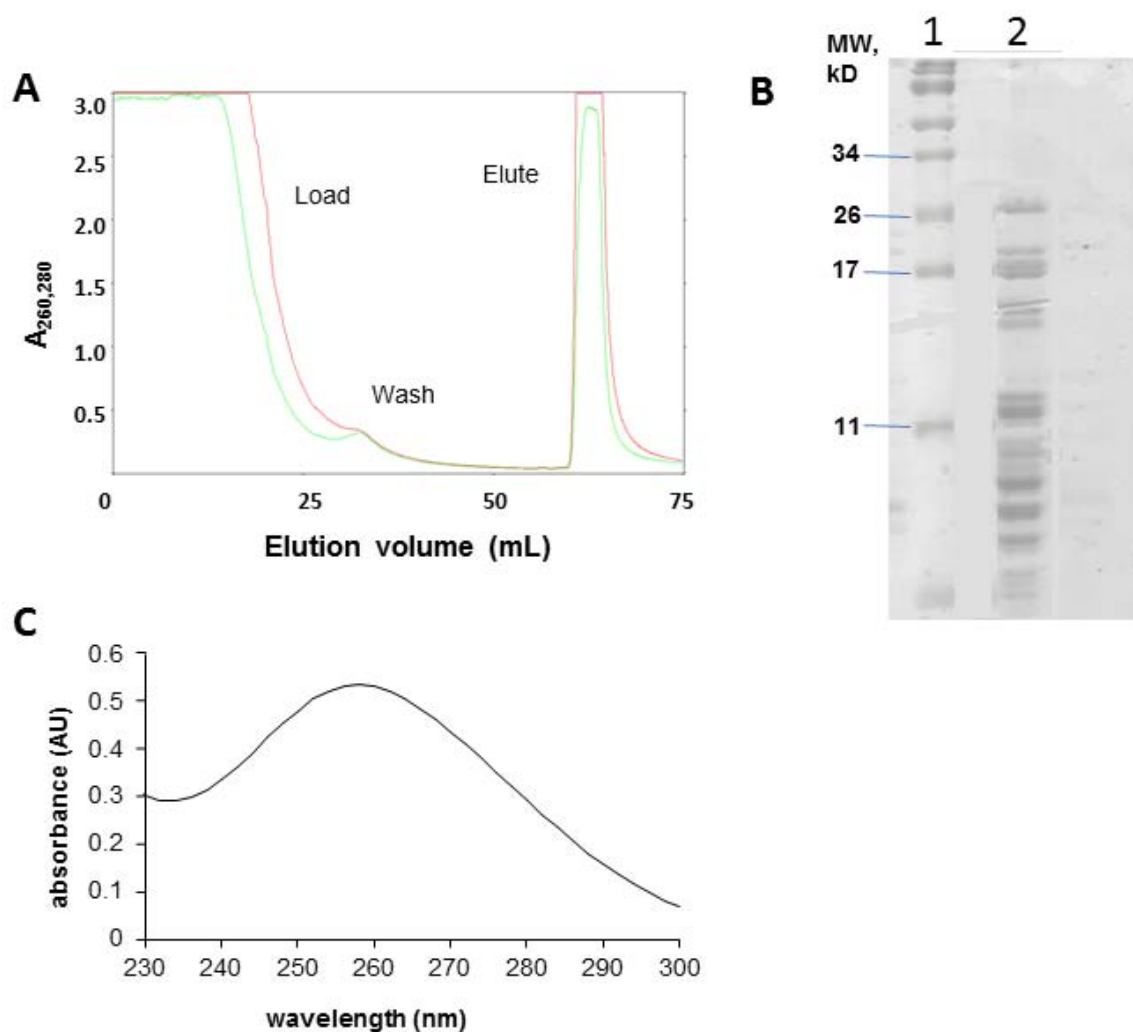


**Figure 2-3. Analysis of ribosomal proteins.** (A) 15% SDS-PAGE. Lanes 1-3 consist of purified, (His)<sub>6</sub>-tagged ribosomal proteins L12, L11, and L10, respectively. (B-D) Circular dichroism spectra of renatured L10 (B), L11 (C), and L12 (D).



### *Purification of ribosomes*

*E. coli* cells of the strain JE28, recently engineered by Ederth et al. (2008), produce endogenous ribosomes which carry a (His)<sub>6</sub> tag at the N-terminus of each copy of the L12 protein, facilitating a simple means of affinity purification of highly active ribosomes (Ederth et al., 2008). With this system, ribosomes can essentially be purified in an analogous manner to (His)<sub>6</sub>-tagged proteins. Ribosomes were purified from *E. coli* strain JE28 as described in the Materials and Methods section. Although rRNA cannot be observed by SDS-PAGE (Figure 2-4B), the profile of ribosomal proteins suggests that both ribosomal subunits are intact and present in purified JE28 ribosomes, based on comparison with the protein profile of 70S ribosomes which were known to be comprised of full 70S particles. A UV-Vis spectrum of a 1:1000-diluted sample of purified ribosomes is shown in Figure 2-4C. The strong absorbance at 260 nm and the ~2:1 ratio of  $A_{260}:A_{280}$  is a characteristic feature of pure ribosomes. Typical yields range from 200-400 pmol of ribosomes per liter of cell culture. Ribosomes were typically purified in 6 L batches, which provided a sufficient quantity to perform numerous biophysical experiments.

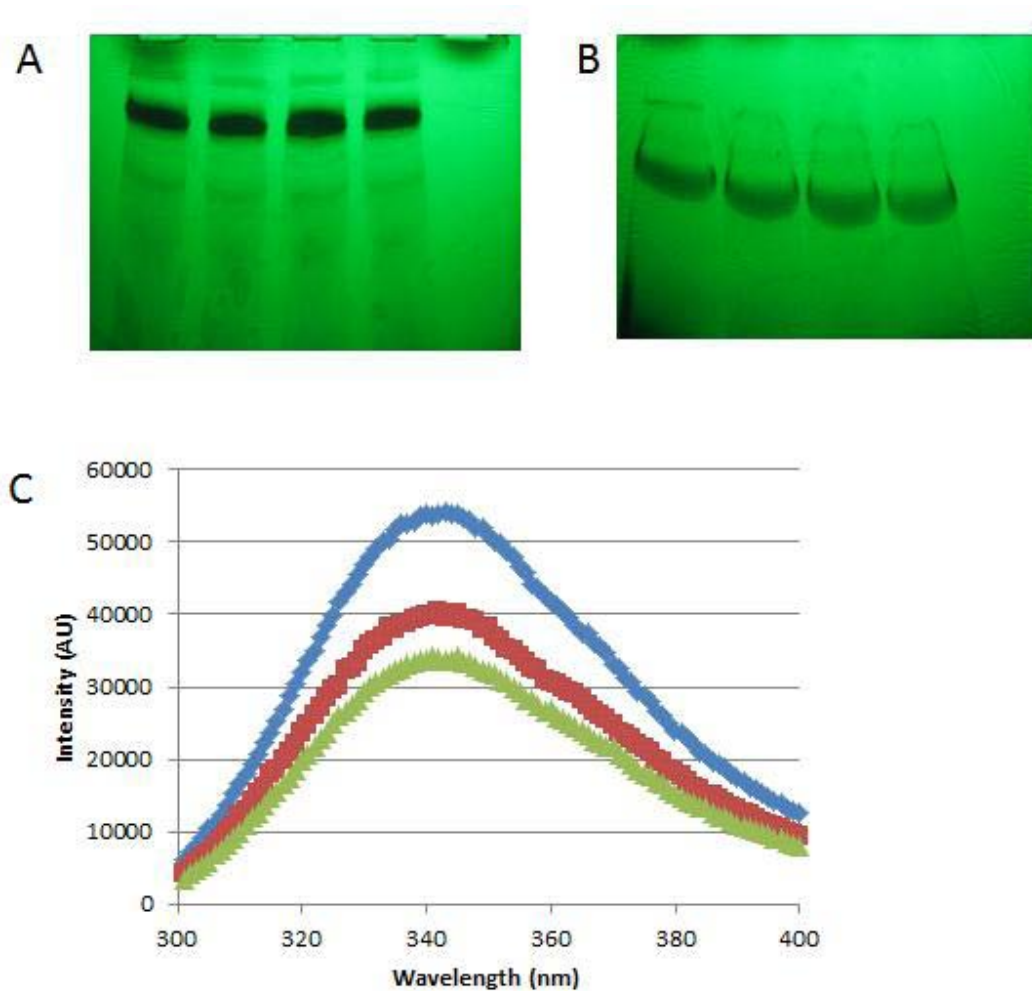


**Figure 2-4. Purification of 70S ribosomes from the *E. coli* strain JE28. (A) Typical elution profile, indicating strong, sharp elution peak. (B) 17 % SDS-PAGE gel of purified 70S ribosomes, showing associated ribosomal proteins; lanes: 1, MW standards; 2, purified ribosomes. (C) UV-Vis spectrum of purified ribosomes, diluted 1000-fold, showing the characteristic strong absorbance at 260 nm and 260:280 ratio of ~2.0, indicative of pure ribosomes.**

### *In vitro transcription of RNA oligoribonucleotides*

The synthetic scheme employed for the in vitro transcription of RNA fragments which mimic the SRL and GAC rRNA utilized a previously described method which has been successful for similar RNA fragments (Milligan et al., 1987; Munishkin and Wool, 1997). It should be noted that special care was taken to avoid contamination by ribonucleases (RNase), which can be a serious problem when performing experiments involving “naked” RNA fragments which are not folded into a stable tertiary structure (Milligan et al., 1987). Upon completion of the transcription reaction there a white precipitate was visible (data not shown), presumably consisting of insoluble magnesium phosphate, a known byproduct of the transcription of RNA (Milligan et al., 1987). Four lines of evidence suggest that these reactions were successful: (1) RNA precipitated from either the SRL or GAC reaction mixture migrates as a single band (Figure 2-5, A-B), suggesting a homogenous product that has not been cleaved by RNase. (2) The products eluted from the excised gel bands absorb very strongly at 260 nm, and the observed  $A_{260}:A_{280}$  ratios of ~2.0 suggest the bands consist of pure nucleic acid (data not shown). (3) Quantification of purified RNA reveals that the product yield generally is at least an order of magnitude higher than the input template DNA, implying that the isolated product consists of transcribed RNA (data not shown). (4) In the presence of in vitro transcribed SRL or GAC RNA, the maximum peak intensity of the fluorescence emission spectrum of EF-G bound to the non-hydrolyzable GTP analog, GDPNP, decreases (Figure 2-5C),

suggestive of the formation of RNA•EF-G•GDPNP complexes, which has been observed previously via gel-retardation assays for the binding of similar small RNA fragments with EF-G•GDPNP (Figure 2-5C) (Munishkin & Wool 1997).



**Figure 2-5.** In vitro transcription of GAC and SRL rRNA mimics. (A) 20% Urea-PAGE of four separate GAC transcriptions. (B) 20% Urea-PAGE of four separate SRL transcriptions. (C) Fluorescence emission spectra of EF-G•GDPNP, blue trace; SRL+EF-G•GDPNP, red trace; GAC+EF-G•GDPNP, green trace. RNA fragments alone emitted a negligible signal.

### *Depletion of L12 from 70S ribosomes*

Treatment of 70S ribosomes in a  $\text{NH}_4\text{Cl}$ /ethanol solution results in a precipitate, consisting of depleted ribosomes, in a solution containing the dissolved extracted protein. Exclusive removal of L12 is strictly dependent on extraction temperature and length of stir time. Harsher conditions employing temperatures higher than 4° C or stir times longer than 10 minutes result in uncontrolled loss of proteins other than L12. Figure 2-6 shows duplicate SDS-PAGE gels, stained with either coomassie brilliant blue or silver nitrate, showing native ribosomes, ribosomes depleted at 4° C, and precipitated protein from the extraction supernatant. Clearly, under these conditions, a single protein matching the MW of L12 is the predominant protein extracted from the ribosome, along with traces of L10. 450 pmol of 70S ribosomes typically yielded about 250 pmol of depleted ribosomes.

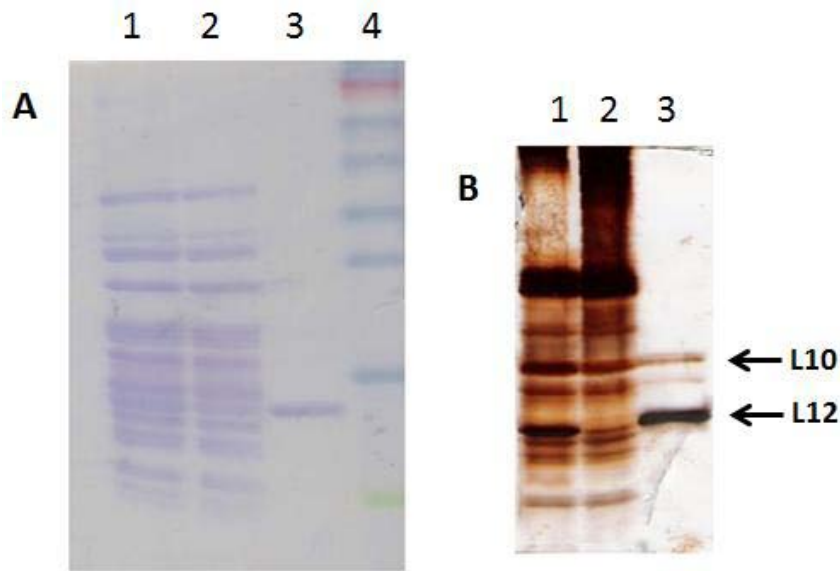
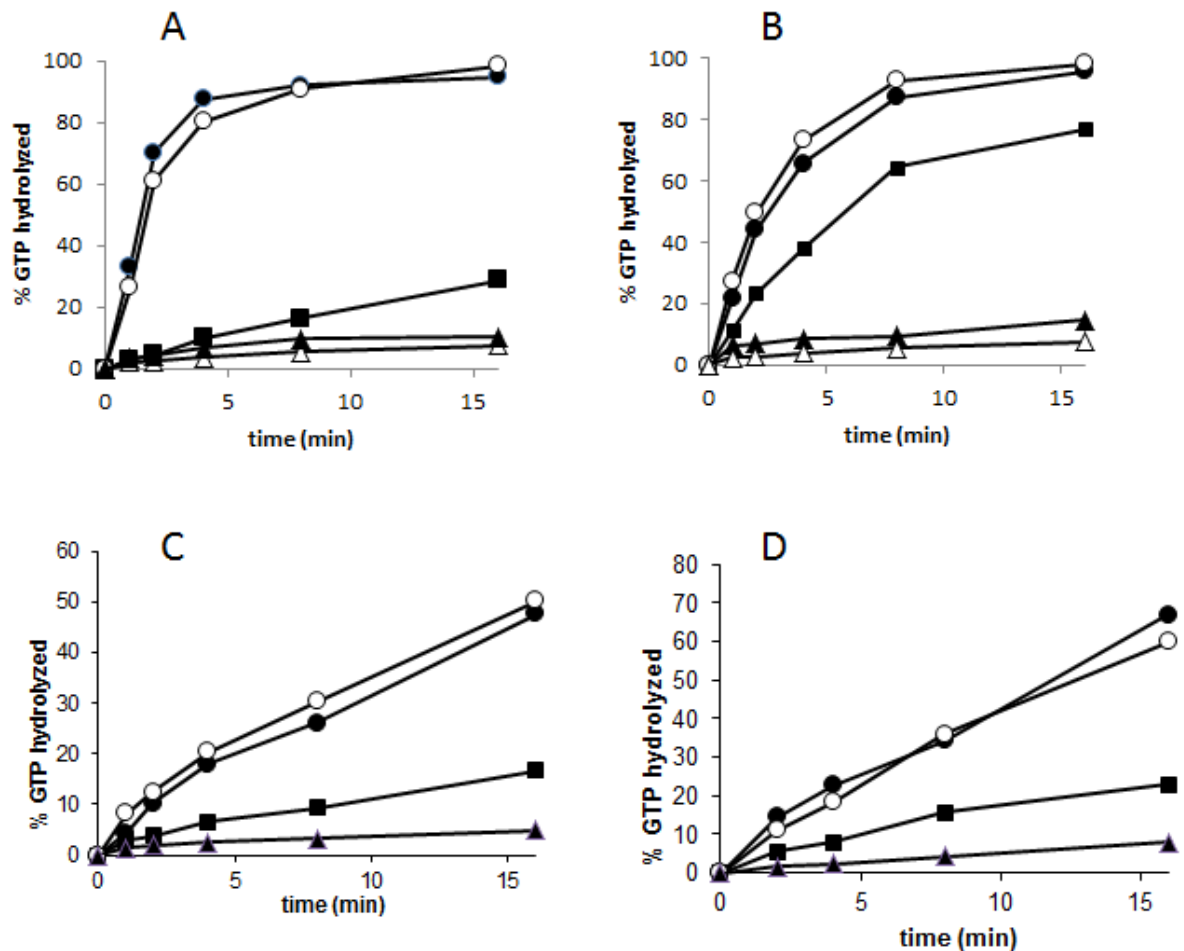


Figure 2-6. Depletion of L12 and trace L10 from 70S ribosomes. (A) Coomassie-stained 17% SDS-PAGE gel. Lanes: 1, wt 70S ribosomes; 2, L12-depleted ribosomes; 3, extracted ribosomal protein; 4, MW standards. (B) Same gel as in (A) but subjected to silver stain.

#### *Effect of L10/L12 depletion on ribosome-dependent GTP hydrolysis*

A GTP hydrolysis assay was developed and used to compare the relative ability of native and L12-depleted ribosomes to stimulate GTPase activity by translation factors. For simplicity, vacant ribosomes (i.e. lacking bound mRNA and tRNAs) were used, as it has been previously demonstrated that vacant ribosomes can stimulate GTP hydrolysis. Results are shown in Figure 2-7. The most apparent observations are that: (1) the loss of L12 decreases the GTP hydrolysis activity of all the translational GTPases. (2) Upon addition of an excess of purified recombinant L12 to depleted ribosomes, full GTP hydrolysis activity is restored.

(3) EF-G shows the most drastic attenuation of GTP hydrolysis activity in the presence of L12-depleted ribosomes. (4) Surprisingly, LepA, which is highly homologous to EF-G, is the least affected by removal of L12 from the ribosome.



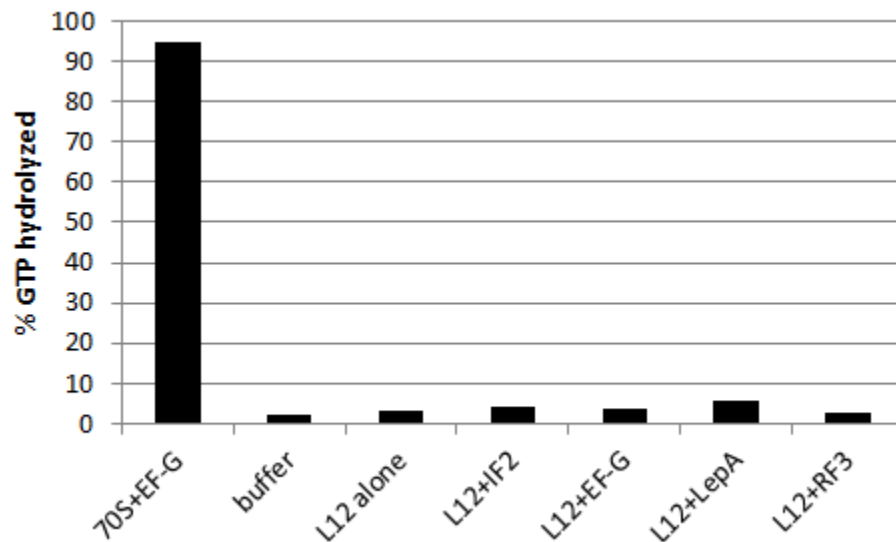
**Figure 2-7. Effect of L12 depletion on ribosome-stimulated GTP hydrolysis by GTPase translation factors. (A) EF-G, (B) LepA, (C) RF3, (D) IF2. GTPase + wt 70S ribosomes, filled circles; GTPase + depleted ribosomes, filled squares; GTPase + reconstituted ribosomes, open circles; GTPase in the absence of ribosomes, filled triangles; ribosomes in the absence of GTPase, open triangles**

*Effect of stalk proteins and oligoribonucleotide rRNA mimics on GTPase activation*

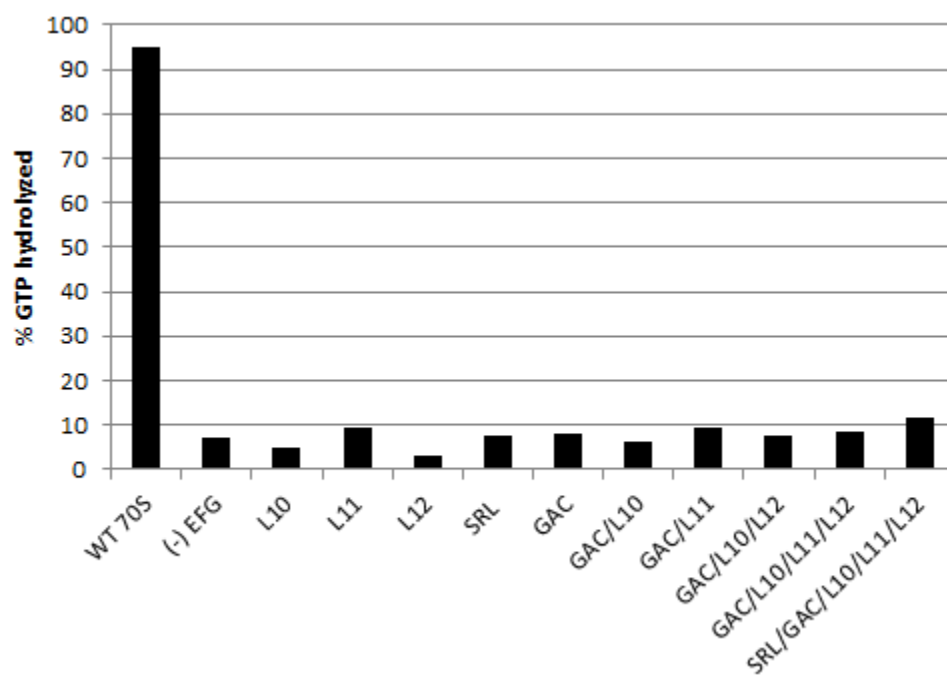
In an oft-cited report by Savelsbergh et al, results of GTP hydrolysis experiments suggested that L12 alone has the capability of strongly stimulating GTP hydrolysis by EF-G, suggesting that L12 may play the role of the ribosomal GAP (Savelsbergh et al., 2000). To test this on the other translational GTPases, the ability of L12 to stimulate GTP hydrolysis by translation factors was assessed, using similar conditions to those of Savelsbergh et al (2000). Surprisingly, no stimulation of GTP hydrolysis by L12 was observed, even with factor EF-G (Figure 2-8). Additionally, incubation of L12 with EF-G•GDPNP did not result in an observable change in the fluorescence emission spectrum, compared to EF-G•GDPNP alone, nor was a signal observed in isothermal titration calorimetry experiments, indicating that no significant binding occurs between L12 and EF-G•GDPNP in these conditions (data not shown). This is in contrast to the observation that purified L12 fully restores the lost GTPase activation activity of L12-depleted ribosomes (Figure 2-7).



To extend this reductionist mode of inquiry to the other ribosomal elements which interact with the translational GTPases, stalk proteins L10 and L11, and in vitro transcribed SRL and GAC rRNA mimics were also tested for stimulation of GTPase activity. Although numerous combinations of these ribosomal components were evaluated, no notable stimulation of GTP hydrolysis activity was observed (Figure 2-9).



**Figure 2-8. Effect of isolated ribosomal protein L12 on GTPase activation of IF2, EF-G, LepA, and RF3.** GTPase (1.0  $\mu$ M) was incubated with L12 (10  $\mu$ M) and [ $\gamma$ - $^{32}$ P]GTP (10  $\mu$ M) at 37° C for 10 minutes, followed by quenching and scintillation counting. The sample labeled 70S+EF-G is a positive control, and a negative control consists of a buffer blank containing only [ $\gamma$ - $^{32}$ P]GTP.



**Figure 2-9. Effect of all isolated ribosomal components, comprising the GTPase binding site, on stimulation of GTP hydrolysis by EF-G.** EF-G (0.5  $\mu$ M) was incubated with the indicated samples and 10  $\mu$ M [ $\gamma$ - $^{32}$ P]GTP at 37° C for 10 minutes, followed by quenching and scintillation counting. (-) EFG is a negative control consisting of EF-G and 10  $\mu$ M [ $\gamma$ - $^{32}$ P]GTP .

## Discussion

*Isolated components which comprise the factor-binding regions of the ribosome do not stimulate the GTP hydrolysis activity of translation factors.*

In spite of remarkable advancements of the past decade in the structural and functional understanding of the ribosome, several aspects of translation remain poorly understood. One enduring mystery is the identity of the component within the complex ribosomal apparatus which is responsible for GTPase activation of translation factors. Our goal here was to use a reductionist approach to identify isolated ribosomal components which are capable of stimulating GTP hydrolysis activity by translational GTPases. This strategy is primarily based on a study by Savelsbergh et al, in which it was reported that isolated ribosomal protein L12 is capable of stimulating significant levels of GTP hydrolysis by EF-G, which the authors suggested could implicate L12 as the elusive GAP of the ribosome (Savelsbergh et al., 2000). Surprisingly, there have not been comprehensive studies published which explicitly explore this phenomenon in more detail, extend it to the other translational GTPases, or that examine the possible roles of other isolated regions of the ribosome in GTPase activation.

In contrast to the report of Savelsbergh, et al, our results do not indicate that isolated L12 is capable of stimulating GTP hydrolysis by EF-G or any of the other translational GTPases (Figure 2-8), even using up to a 50-fold excess of L12 over GTPase and monitoring the reaction over an extended period of time of up

to 30 minutes (data not shown). This negative result is in agreement with an early investigation by Donner et al (Donner et al., 1978). There are several possible reasons for the discrepancy between our results and those of Savelsbergh, et al. One possibility is that the recombinant L12 we have expressed and purified is either insufficiently pure or improperly folded. However, SDS-PAGE and circular dichroism analysis indicates that our L12 is pure and in a folded state (Figure 2-3). Also, importantly, our recombinantly purified L12 is capable of completely restoring lost GTP hydrolysis activity to L12-depleted ribosomes (Figure 2-7), which casts doubt on the notion that our negative result is due to misfolding or contamination of L12. However, the result of Savelsbergh et al. could very likely be due to trace contamination of L12 by ribosomes or some other ribosomal element such as L10, which binds to L12 with high affinity (Iben and Draper, 2008). This possibility is highlighted by the fact that the purification method used by the Savelsbergh et al merely employs ultracentrifugation of the cell lysate to remove ribosomes, followed by chromatographic steps which explicitly do not involve chemical denaturants (Oleinikov et al., 1993). We found these conditions insufficient for complete removal of ribosomes from L12 preparations (data not shown); our results indicate that full removal of ribosomes is only accomplished by a combination of denaturing conditions and ultracentrifugation.

Another possible contributing factor to our results in experiments testing GTPase activation by L12 relates to the question of how tightly L12 can bind to EF-G in

solution. Savelsbergh et al reported an estimated  $K_d$  of 10  $\mu$ M for the binding of L12 to EF-G, based on intrinsic tryptophan fluorescence measurements. However, if the L12 purified by Savelsbergh et al. was contaminated by trace ribosomes, then this value is questionable because any observed signal arising from the high affinity of EF-G binding to the ribosome would erroneously be interpreted as being due to L12 binding to EF-G, which would result in an artificially low  $K_d$  value. Also, an NMR study investigating the interaction between L12 and the translational GTPases reported much higher binding affinities, in the range of 0.2 mM-2.5 mM (Helgstrand et al., 2007). However, the NMR-derived  $K_d$  values were determined in the absence of guanine nucleotides, while, although not explicitly stated in the text, the Savelsbergh estimation was likely determined in the presence of GTP or GDPNP. Clearly, more studies will be necessary to unambiguously determine the binding affinity of L12 for translational GTPases in the presence and absence of guanine nucleotides. This data will be crucial for the proper design of future experiments.

It is known that the consensus binding site for translational GTPases consists of two distinct regions. One, the GAC, consists of a conserved region of rRNA which is in turn bound to both ribosomal protein L11 as well as to the complex L10(L12)<sub>4</sub> (Figures 1-14 and 1-16). The other region consists of the highly conserved SRL rRNA (Figures 1-14 and 1-15). We sought to replicate these important regions in vitro and subsequently test these complexes for stimulation of GTP hydrolysis. This seemed to be a realistic goal, as it has been shown

previously that the complex L10(L12)<sub>4</sub>, as well as ribosomal protein L11, bind to an in vitro transcribed RNA fragment which mimics the GAC rRNA (Diaconu et al., 2005; Wimberly et al., 1999). Also, RNA fragments which mimic the SRL or GAC rRNA independently bind to EF-G with affinities that approach that of EF-G-ribosome binding (Munishkin and Wool, 1997) and crystal structures show that these mimics adopt native-like tertiary structure (Figures 1-15 and 1-17).

Employing EF-G as the canonical translational GTPase, we systematically tested each isolated component of both GTPase binding regions, as well as complexes formed in vitro by incubating the components together. Unfortunately, no stimulation of translation factor GTP hydrolysis was observed for any of these ribosomal components (Figure 2-9). There are several plausible explanations for these results: (1) Although circular dichroism spectra reveal that the ribosomal proteins L10, L11 and L12 are folded (Figure 2-3), it is possible that their tertiary structures in solution are different than their physiologically relevant conformations on the ribosome, perhaps due to the absence of influential interactions from peripheral ribosomal elements. (2) The SRL and GAC rRNA mimics may not be properly purified or folded into their native tertiary structures, in spite of the evidence shown in Figure 2-5 which suggests that these rRNA mimics are capable of binding to EF-G. (3) This reductionist mode of inquiry may be an inappropriate means of investigating the ribosomal component responsible for GTPase activation, especially if the responsible component requires the structural context of the ribosome for correct positioning of vital functional groups.

*Depletion of L12 from the 70S ribosome has a varying effect on GTP hydrolysis activity of GTPase translation factors*

Several previous reports have shown that the depletion of L12 from the ribosome results in the decreased ability to interact with the translational GTPases (Brot et al., 1974; Mohr et al., 2002; Savelsbergh et al., 2005). However, the relative effect of L12 depletion on the individual translational GTPases is difficult to discern from these studies because each report has generally only focused on a single GTPase and, importantly, experimental conditions for these studies, such as the composition of the L12 extraction buffer, methods of L12 purification, temperature, and concentration of components, have differed significantly. Also, the effect of L12 depletion on the activity of the newly discovered factor LepA has not been described. Therefore, we sought to examine the effect of L12 depletion of vacant ribosomes on the GTPase activity of all translation factors, using a standard assay which could allow direct comparison in a straightforward manner.

Our results confirm the notion that the removal of L12 from native 70S ribosomes results in loss of GTP hydrolysis activity, although the magnitude of this change differs significantly between GTPases (Figure 2-7). In all cases, lost GTPase activity is completely restored by pre-incubation of depleted ribosomes with a 5-fold excess of purified L12, which demonstrates the integrity of our L12 purification and refolding process. In the presence of native ribosomes, IF2 and RF3 hydrolyze GTP at a significantly slower rate than EF-G and LepA, which is

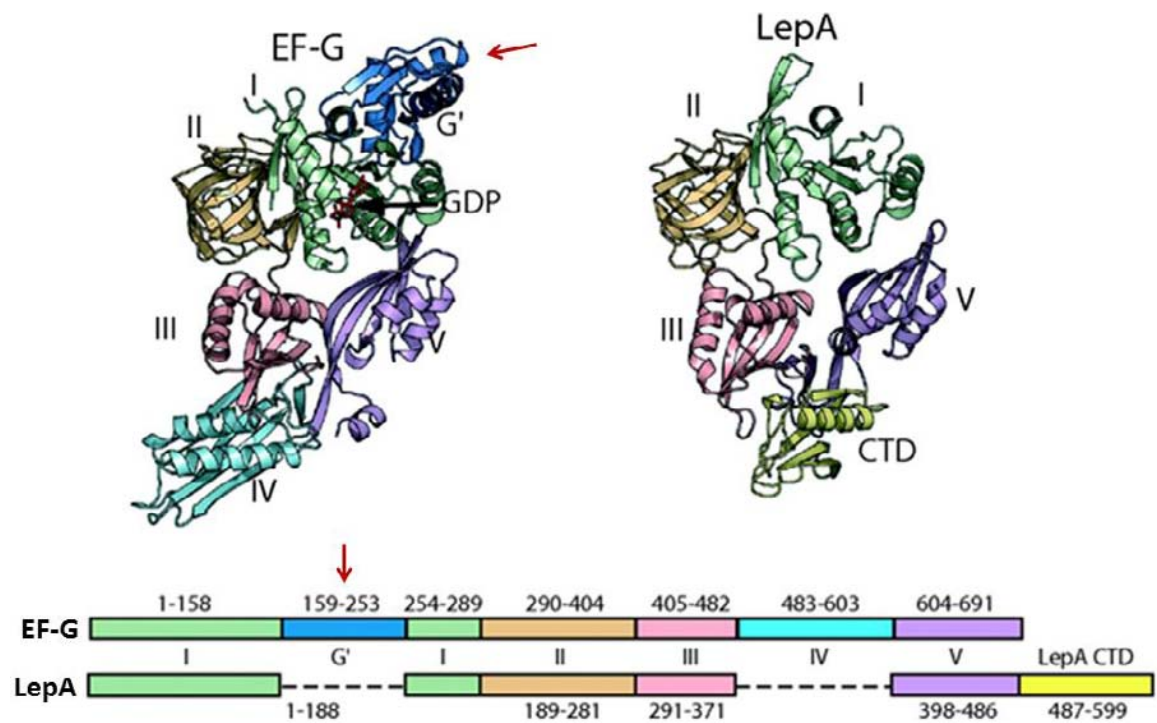
consistent with the observation that vacant ribosomes are not an ideal substrate for IF2 and RF3 (Roll-Mecak et al., 2004; Zavialov et al., 2001). Surprisingly, L12 depletion has a significantly smaller effect on the GTP hydrolysis activity of LepA than for EF-G, despite the strong homology between EF-G and LepA and nearly identical levels of activity in the presence of native ribosomes. This unexpected result is difficult to explain with the available biochemical and structural data.

#### *Role of the G' module of EF-G*

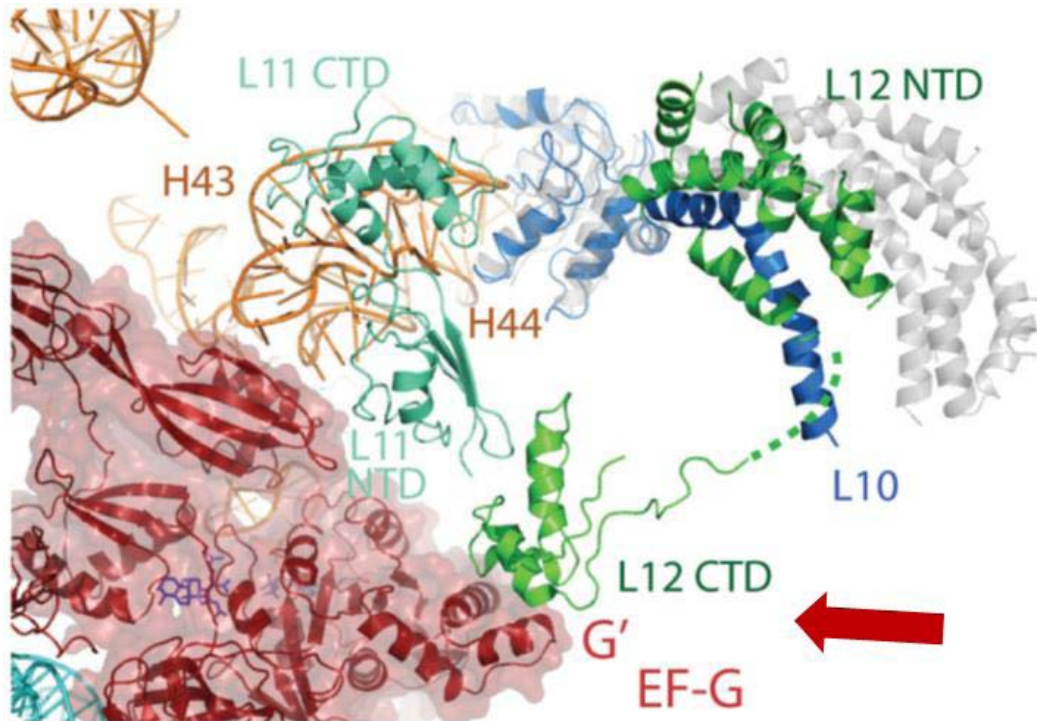
An attempt to understand the lack of a similar response to L12 depletion between EF-G and LepA prompted a further analysis of the structural differences between EF-G and LepA. It is notable that within the G domain of EF-G there is a novel subdomain called G' which extends from the periphery of the G domain and is not present in the LepA structure (Evans et al., 2008). This G' module is not observed in LepA (Figure 2-10). The functional role of this G' domain has not been elucidated. Interestingly, cryo-EM and x-ray crystal structures of EF-G•GDP•ribosome complexes have revealed that one of the four L12 CTDs contacts the G' domain (Figure 2-11) (Datta et al., 2005; Gao et al., 2009). However, there is no structural or biochemical evidence for this interaction in the pretranslocation complex consisting of EF-G in the GTP state. It has been suggested that interactions between the L12 CTD and the G' domain of EF-G may cause conformational changes which accelerate events after GTP



hydrolysis by EF-G, such as phosphate release and factor turnover (Savelsbergh et al., 2005). This could explain the apparent hypersensitivity of EF-G to the presence of L12 for optimal function. However, RF3 also contains a G'-like domain but does not exhibit the same degree of hypersensitivity as EF-G (Gao et al., 2007). It is possible that L12 could simultaneously act as a generic factor recruitment module as well as an EF-G-specific stimulator of factor turnover. Future investigations will be aimed at elucidating the role of the G' domain by using genetic engineering techniques to delete or modify the G' domain of EF-G as well as to introduce a G' module to LepA, followed by assessment of the effects of these alterations on GTPase activity.



**Figure 2-10. Structural comparison of EF-G and LepA.** LepA shares several homologous domains with EF-G, but lacks the G' domain (red arrows). Adapted from Evans, et al. (2008).



**Figure 2-11.** The L12 CTD contacts the G' domain of EF-G (red arrow), as observed in a crystal structure of EF-G bound to the ribosome in the posttranslocational, GDP state. Adapted from Gao et al., 2009.

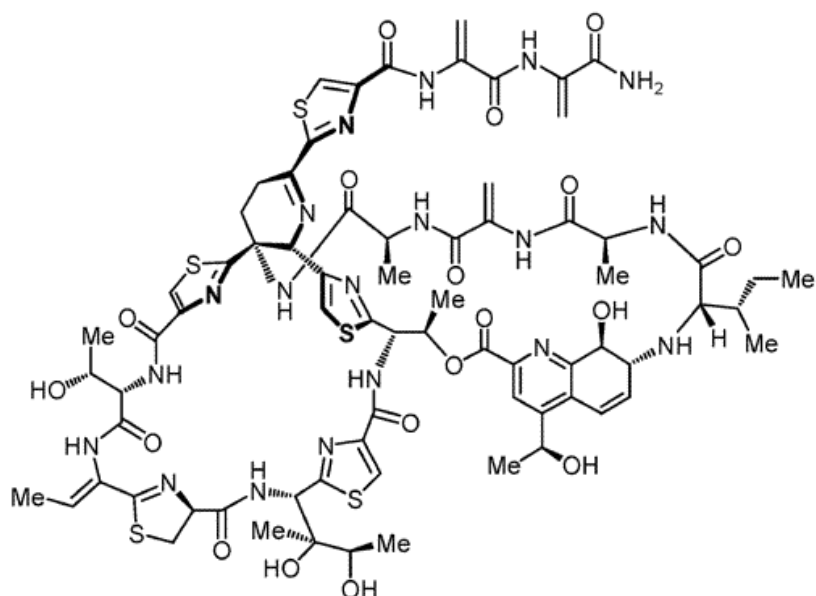
## **Chapter 3 – Functional investigations of the antibiotic thiostrepton**

### **Introduction**

The ribosome is a major cellular target for naturally occurring antibiotics. In fact, the majority of clinically available antibiotics function by specifically binding to the prokaryotic ribosome in such a manner which results in the inhibition of a particular step of protein synthesis (Douthwaite, 1992). The past decade has brought considerable progress in the understanding of ribosome function due to the determination of several high resolution x-ray crystal structures of ribosomes in complex with various antibiotics. These structures, along with biochemical data, have provided a wealth of information about the mode of action of antibiotics and have revealed that their usual targets are functionally important regions of rRNAs, such as the decoding center on the small subunit and the peptidyltransferase center on the large subunit (Harms et al., 2008).

One particularly well-studied antibiotic which inhibits translation is thiostrepton (Figure 3-1), a highly modified macrocyclic peptide natural product (Schoof et al., 2010). Discovered in 1955, thiostrepton is a natural product of the microorganism *Streptomyces azureus* (Donovick et al., 1955). The 50S ribosomal subunit was implicated as the site of action of thiostrepton in 1970 (Weisblum and Demohn, 1970). Shortly thereafter, the first x-ray crystal

structure of thiostrepton was determined (Anderson et al., 1970). Subsequently, it was reported that thiostrepton inhibits the binding and ribosome-mediated GTP hydrolysis activity of EF-G (Highland et al., 1971) and also that thiostrepton itself binds with high affinity to a complex of 23S rRNA and ribosomal protein L11, a region which lies near the binding site of EF-G (Thompson et al., 1979). From these and other experiments a model for the action of thiostrepton was proposed in which thiostrepton, upon binding to the 23S rRNA-L11 complex, inhibits the binding of EF-G to the ribosome. However, this paradigm was challenged by a report from Rodnina et al, which re-analyzed the function of thiostrepton using rapid stopped-flow fluorescence techniques (Rodnina et al., 1999). Based on the results of Rodnina et al, it was concluded that thiostrepton does not prevent the binding and GTP hydrolysis of EF-G, but inhibits later steps such as the release of inorganic phosphate. This study is now the predominantly cited model for the action of thiostrepton (Bowling et al., 2008; Gao et al., 2009; Garcia-Ortega et al., 2010; Gonzalez et al., 2007). However, there have also been conflicting reports which reaffirm the classical model of thiostrepton inhibiting EF-G binding (Cameron et al., 2002).



**Figure 3-1. Thiostrepton. Adapted from Nicolaou et al. (2005a)**

Although thiostrepton is currently utilized primarily as a tool for the study of ribosome function, several recent developments have renewed interest in the possible therapeutic use of thiostrepton. Remarkably, it has been found that thiostrepton is a potent inhibitor of the growth of the malarial parasite *Plasmodium falciparum* (Clough et al., 1997; Goodman et al., 2007; McConkey et al., 1997), a parasite which annually infects more than 200 million individuals and is responsible for one to three million deaths every year (Snow et al., 1999). The total synthesis of thiostrepton has been published (Nicolaou et al., 2005b), and it has been discovered that small fragments of thiostrepton retain inhibitory activity against *P. falciparum* (Nicolaou et al., 2005a; Schoof et al., 2010). However, in order to fully exploit the anti-malarial activity of thiostrepton it is imperative that a

consensus is reached as to the precise inhibitory mechanism of thiostrepton on the ribosome.

## **Research Aims**

The goal of the presented research is to resolve questions about how thiostrepton inhibits the activity of the translation factor EF-G. Experiments have been designed to investigate: (1) The effects of thiostrepton on ribosome-binding and GTP hydrolysis by EF-G and the highly homologous translation factor LepA; and (2) The role of domain V of EF-G and LepA in the inhibitory mechanism of thiostrepton.

## Materials and Methods

### *Mutagenesis*

The gene encoding and EF-G mutant lacking domains IV and V (EF-G $\Delta$ 4,5) was constructed via introduction of a stop codon immediately upstream from the sequence encoding domains IV and V of EF-G by PCR mutagenesis. The mutant gene was then cloned into expression vector pSV, overexpressed, and purified as described in the Chapter 2 Materials and Methods section.

### *GTP hydrolysis assays*

GTP hydrolysis experiments were carried out as described in the Chapter 2 Materials and Methods section, with the following exceptions. Where indicated in figure legends, ribosomes were pre-incubated with thiostrepton at 37° C for 10 minutes. For timecourse measurements, final concentrations of components were 0.2  $\mu$ M ribosomes, 0.5  $\mu$ M GTPase, 10  $\mu$ M [ $\gamma$ -<sup>32</sup>P]GTP, and 10  $\mu$ M thiostrepton. For the single-timepoint dose-response experiments, final concentrations of components were identical, except that the concentration of thiostrepton used was varied as noted in Figure 3-3. Additionally, all buffers contained 2% v/v dimethyl sulfoxide (DMSO) to assist in the solubilization of thiostrepton.



### *Ribosome binding assay*

To assess the binding of GTPase translation factors to the ribosome, 60  $\mu$ L samples containing 1.0  $\mu$ M ribosomes, 4.0  $\mu$ M GTPase, 1 mM GDPNP, and 10  $\mu$ M thiostrepton (as indicated in the legend to Figure 3-4) were first preincubated at 37° C for 20 minutes in GTPase reaction buffer supplemented with 2% DMSO. 1 mL Sephacryl-300 size exclusion resin was added to spin columns and washed with 1 mL GTPase reaction buffer by pipetting the buffer onto the resin and centrifuging the spin column for 1 minute in an International Clinical Centrifuge at 2000 rpm. The flow-through wash buffer was discarded and the wash was repeated twice, with the final wash buffer also containing 1 mM GDPNP. Samples were then added directly to the resin bed and the resin was incubated for 45 seconds followed by elution via centrifugation in a clinical centrifuge at 2000 rpm for 2 minutes. Proteins in eluted samples were then precipitated with cold acetone and analyzed on 10% SDS-PAGE gels.

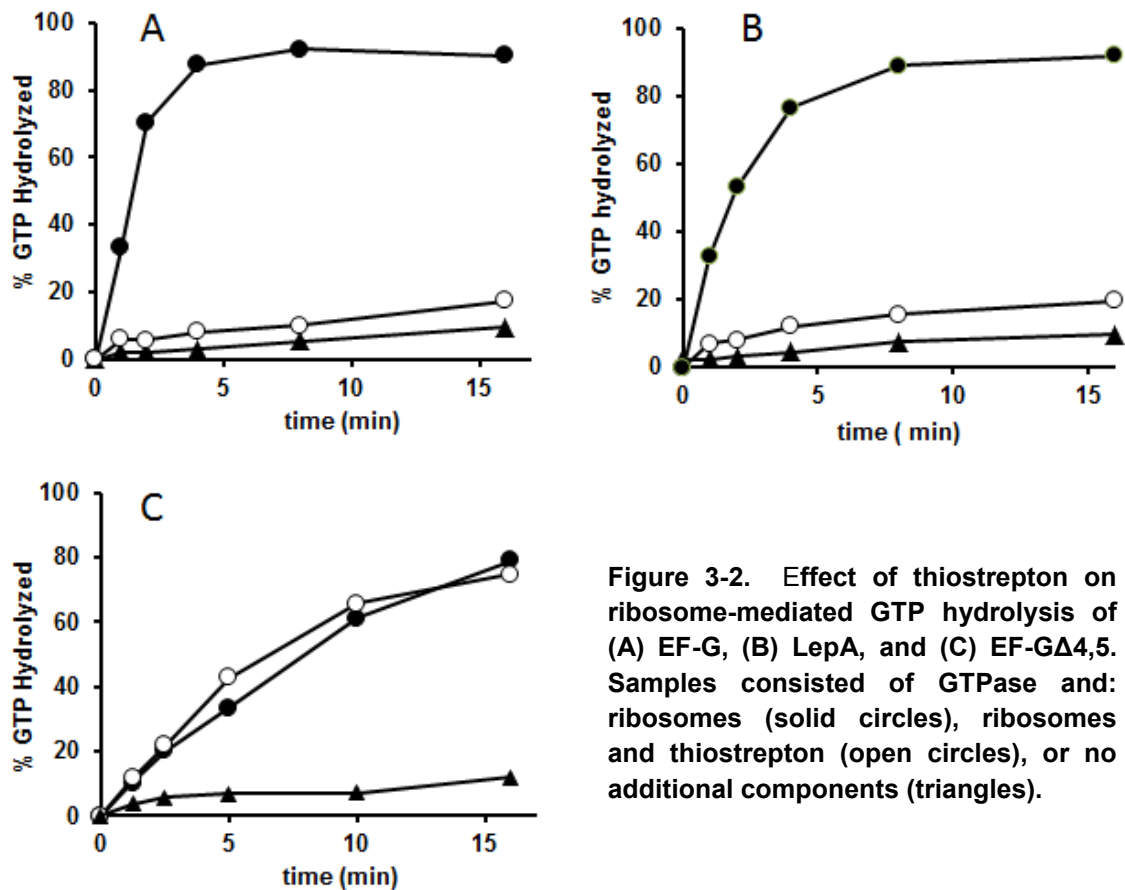
## Results

### *Effect of thiostrepton on the GTP hydrolysis activity of EF-G and LepA*

Vacant 70S ribosomes were incubated with thiostrepton at 37° C and were tested for their ability to stimulate GTP hydrolysis by EF-G and LepA (Figure 3-2, A-B). Conditions for the experiments were deliberately chosen to replicate those reported by Rodnina et al., with minor exceptions (see Materials and Methods). Notably, it was found that thiostrepton forms insoluble precipitates at the concentration used by Rodnina et al. (100  $\mu$ M), so we chose to use a concentration of 10  $\mu$ M, which does not cause precipitate formation. In the absence of thiostrepton, GTP is rapidly hydrolyzed by EF-G and LepA in the presence of vacant ribosomes, with over 90% of the GTP being hydrolyzed within the first four minutes of the experiment for both EF-G and LepA. Neither ribosomes, thiostrepton, nor factors alone resulted in significant GTP hydrolysis. In the presence of thiostrepton, negligible GTP hydrolysis was observed for both GTPases throughout the duration of the time course.

To examine the relative potency of thiostrepton, concentrations of ribosomes and factors were held constant and single endpoint measurements of hydrolyzed GTP were recorded as a function of increasing thiostrepton concentration (Figure 3-3). These experiments reveal thiostrepton to be a very potent inhibitor of GTP hydrolysis by EF-G and LepA. GTPase activity is drastically attenuated as the concentration of thiostrepton approaches 0.2  $\mu$ M and activity is completely

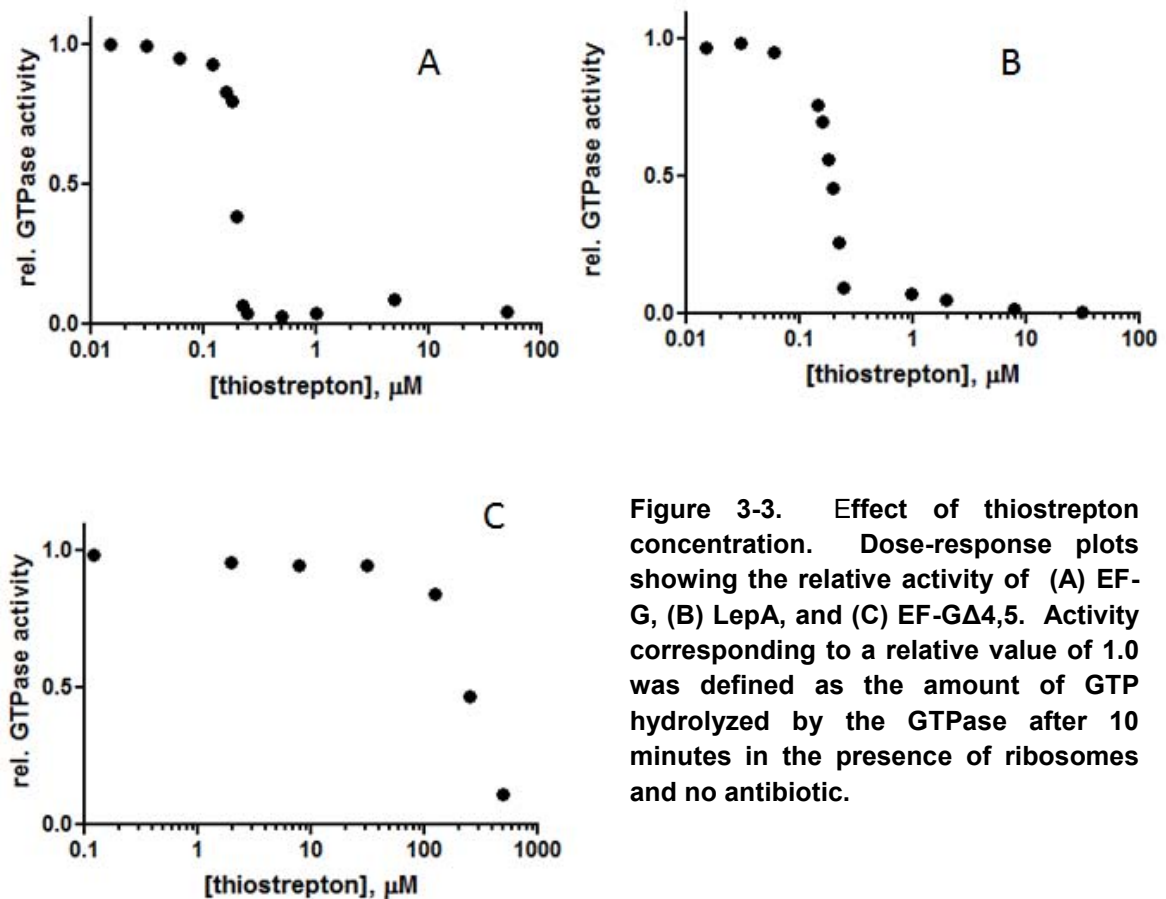
suppressed when the concentration of thiostrepton is raised above 0.2  $\mu\text{M}$ . Since the concentration of ribosomes used in these experiments was 0.2  $\mu\text{M}$ , this result indicates that a 1:1 molar ratio of thiostrepton to ribosomes is sufficient for complete inactivation of EF-G and LepA GTP hydrolysis activity.



**Figure 3-2.** Effect of thiostrepton on ribosome-mediated GTP hydrolysis of (A) EF-G, (B) LepA, and (C) EF-G $\Delta$ 4,5. Samples consisted of GTPase and: ribosomes (solid circles), ribosomes and thiostrepton (open circles), or no additional components (triangles).

### *Effect of thiostrepton on a mutant EF-G lacking domains IV and V*

To test the effect of domains IV and V of EF-G on GTP hydrolysis and inhibition by thiostrepton, a mutant clone of EF-G lacking domains IV and V (EF-G $\Delta$ 4,5) was constructed, expressed in *E. coli*, and purified. Figure 3-2C shows the results of a time course experiment testing the effect of thiostrepton on GTP hydrolysis. Notably, EF-G $\Delta$ 4,5 exhibits ribosome-dependent GTP hydrolysis activity. Although the apparent rate of GTP hydrolysis by EF-G $\Delta$ 4,5 is slower than that of full-length EF-G, the reaction is not observably affected by the presence of thiostrepton. Furthermore, the dose-response plot in Figure 3-3C reveals that even when the concentration of thiostrepton is increased to a 500-fold excess over ribosomes (100  $\mu$ M thiostrepton), there is a negligible effect on the GTP hydrolysis activity of EF-G $\Delta$ 4,5. As mentioned above, at concentrations above 100  $\mu$ M we found thiostrepton to be insoluble and therefore interpret any decreases in activity above this concentration to be due to nonspecific interactions.



**Figure 3-3.** Effect of thiostrepton concentration. Dose-response plots showing the relative activity of (A) EF-G, (B) LepA, and (C) EF-G $\Delta$ 4,5. Activity corresponding to a relative value of 1.0 was defined as the amount of GTP hydrolyzed by the GTPase after 10 minutes in the presence of ribosomes and no antibiotic.

### *Effect of thiostrepton on GTPase binding*

To assess the effect of thiostrepton on ribosome-factor binding, we employed an empirically developed ribosome-binding assay (Figure 3-4). A goal of the assay is to avoid harsh conditions which may prematurely dissociate factors from ribosomes and lead to erroneous results, which is a criticism of ribosome binding experiments which employ techniques such as ultracentrifugation (Cameron et

al., 2002; Gao et al., 2009). The method employed here involves gently centrifuging ribosome-factor complexes through size-exclusion resin at low speed, conditions which should not cause premature dissociation of ribosome-bound factors. Conditions were empirically adjusted such that ribosomes with bound factors are allowed to elute, while any unbound factors are retained on the resin. Eluted protein fractions are subsequently precipitated with acetone and subjected to SDS-PAGE analysis.

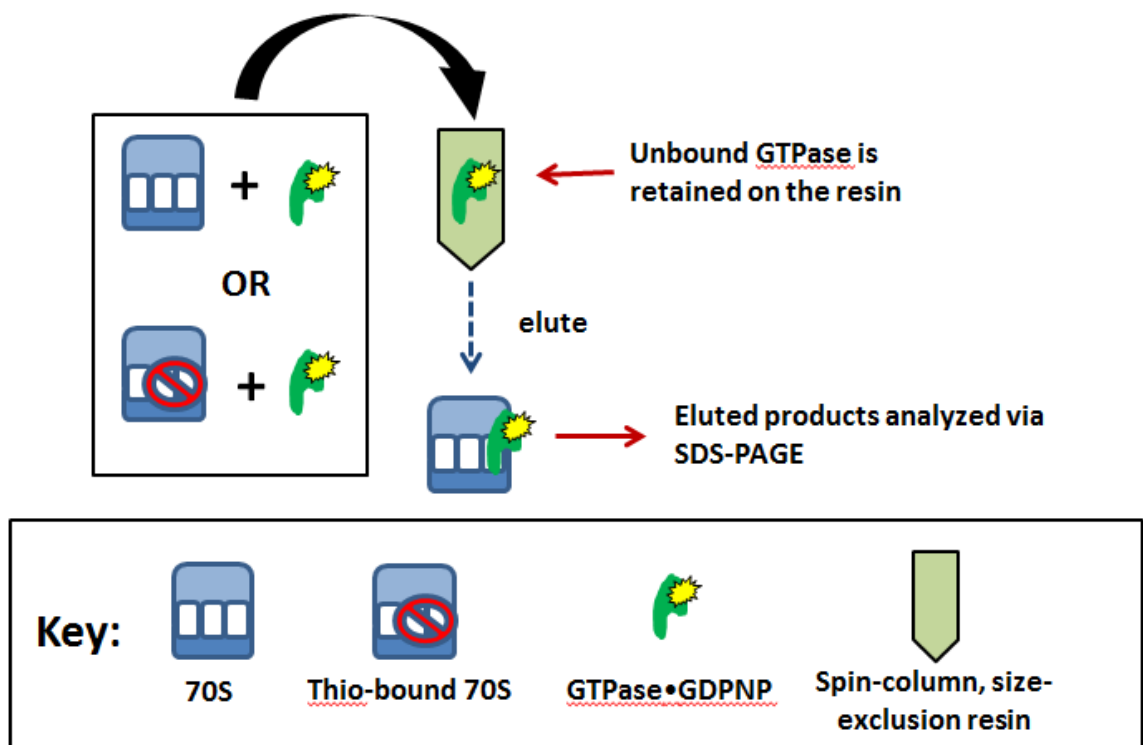
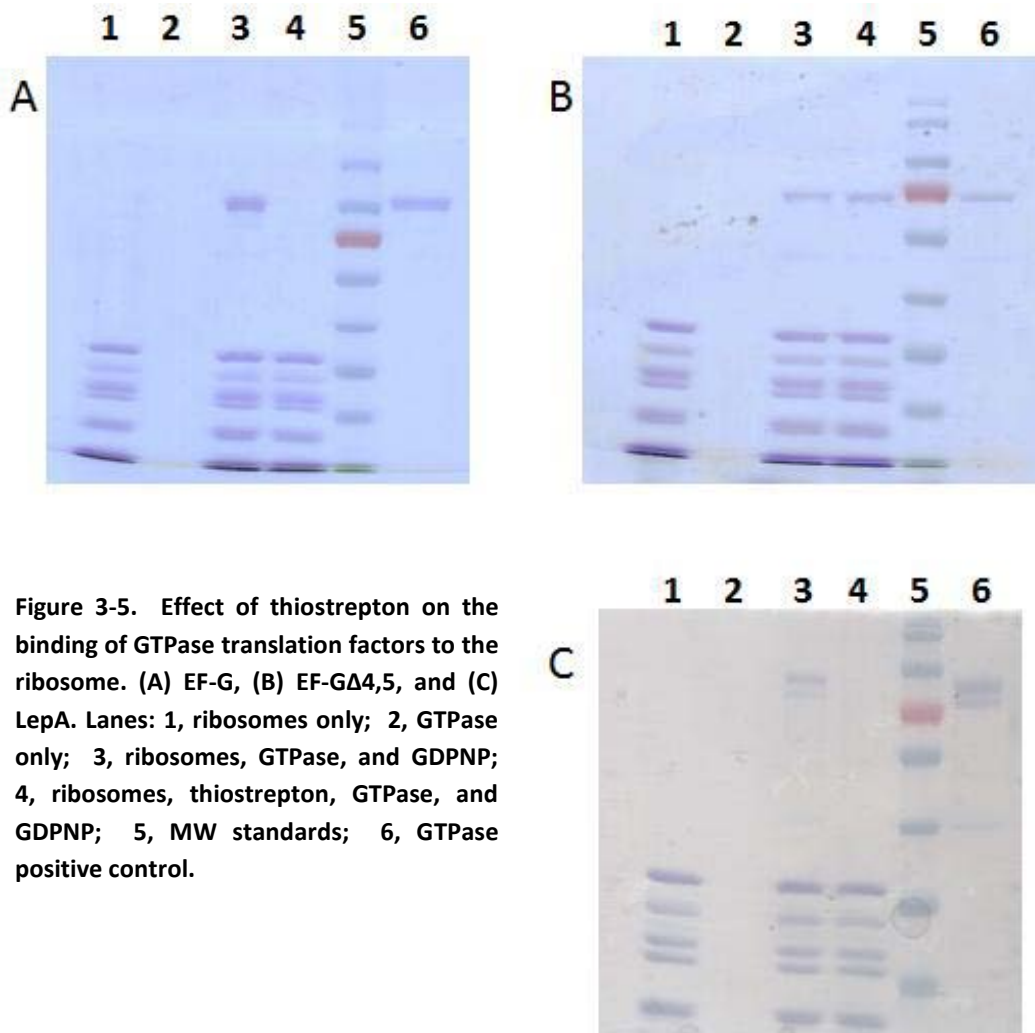


Figure 3-4. Overview of the ribosome binding assay employed in this study.

Figure 3-5 shows the results of binding experiments, in the presence and absence of thiostrepton. The binding of both EF-G•GDPNP and LepA•GDPNP is inhibited by the presence of thiostrepton. However, there is no apparent effect on the binding of EF-G $\Delta$ 4,5•GDPNP to ribosomes (Figure 3-5B), which is consistent with its observed insensitivity to thiostrepton in GTP hydrolysis experiments (Figures 3-2 and 3-3).



**Figure 3-5.** Effect of thiostrepton on the binding of GTPase translation factors to the ribosome. (A) EF-G, (B) EF-G $\Delta$ 4,5, and (C) LepA. Lanes: 1, ribosomes only; 2, GTPase only; 3, ribosomes, GTPase, and GDPNP; 4, ribosomes, thiostrepton, GTPase, and GDPNP; 5, MW standards; 6, GTPase positive control.

## Discussion

### *Inhibition of GTPase activity and ribosome binding by thiostrepton*

Thiostrepton, a macrocyclic thiopeptide antibiotic which has shown promising antimalarial activity, inhibits the function of the GTPase translation factor EF-G (Highland et al., 1971). Results from a series of experiments performed by Rodnina et al. culminated in the formation of a model in which thiostrepton functions not by inhibiting ribosome-binding or GTP hydrolysis by EF-G, but by inhibiting steps after GTP hydrolysis, such as the release of inorganic phosphate and factor turnover (Rodnina et al., 1999). We report several lines of evidence which are inconsistent with this model. Rodnina et al reported data which showed that, while thiostrepton decreased the rate of ribosome-dependent GTP hydrolysis by EF-G, the full-extent of hydrolyzed GTP was still reached within the time course of the experiment. We repeated this experiment using identical conditions; however, we found that the high concentration of thiostrepton used by Rodnina et al. (100  $\mu$ M) resulted in the formation of insoluble precipitates, so we used a concentration of 10  $\mu$ M thiostrepton. The apparent insolubility of thiostrepton at concentrations used by Rodnina et al. has also been reported elsewhere (Cameron et al., 2002). Our results indicate that the presence of thiostrepton results in the complete inhibition of GTP hydrolysis by both EF-G and a recently described, closely related translation factor, LepA. We also show in dose-response experiments that thiostrepton is a very potent inhibitor of GTP



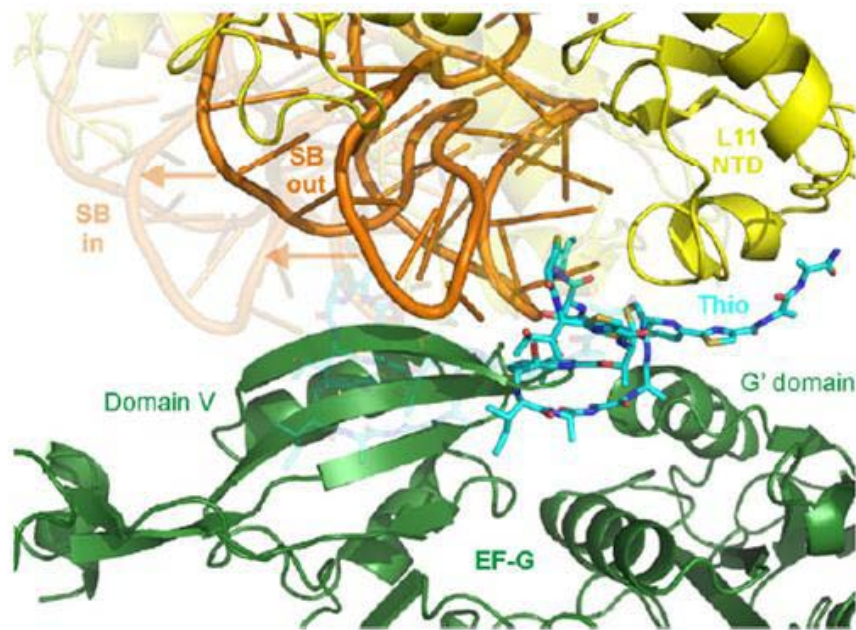
hydrolysis by both EF-G and LepA; a 1:1 molar ratio of thiostrepton to ribosomes is sufficient to completely inactivate the GTPase-activation properties of the ribosome. This is consistent with the finding that thiostrepton binds to the ribosome with a  $K_d$  of approximately  $2 \times 10^{-7}$  M (Thompson and Cundliffe, 1991).

Rodnina et al. also interpret their experimental results to indicate that thiostrepton inhibits the release of EF-G from the ribosome. However, they also show the results of chemical footprinting experiments which indicate that thiostrepton prevents the protection, by EF-G, of 23S rRNA bases within the SRL from modification by base-specific reagents (Rodnina et al., 1999). They claim that this observation is possibly due to thiostrepton stabilizing a unique conformation of EF-G on the ribosome which does not make molecular contacts with the SRL. This interpretation is remarkable, as the available biochemical and structural data (Gao et al., 2007; Moazed et al., 1988), implies that the lack of any protection of the SRL by EF-G in the presence of thiostrepton is likely due to inhibition of factor binding by thiostrepton. Following the report of Rodnina et al. (1999), a study by Cameron et al. demonstrated that thiostrepton inhibits the binding of EF-G to the ribosome (Cameron et al., 2002). However, it was later argued that the lack of EF-G binding observed by Cameron et al. could be due to overly harsh experimental conditions, such as ultracentrifugation, which could cause premature dissociation of EF-G from the ribosome-thiostrepton complex (Gao et al., 2007). To address this concern, we optimized a gentler binding assay which involved injecting ribosome complexes onto spin-chromatography columns

harboring size exclusion resin (Figure 3-4), followed by low-speed centrifugation for a short, empirically determined amount of time which allows ribosomes and bound factors to elute yet retains unbound factors on the resin. Proteins in the eluent are then precipitated with acetone and subjected to SDS-PAGE analysis to determine binding. While at odds with the results of Rodnina et al., our results (Figure 3-5) are in agreement with other studies (Cameron et al., 2002; Thompson et al., 1979) and indicate that thiostrepton completely inhibits the binding of EF-G and LepA to vacant 70S ribosomes.

#### *Role of domain V of EF-G and LepA in thiostrepton activity*

Thiostrepton binds to the GTPase-associated center GAC rRNA of 50S ribosomal subunit, in a cleft between 23S rRNA and ribosomal protein L11 (Harms et al., 2008; Thompson and Cundliffe, 1991). A study in which an X-ray crystal structure of thiostrepton bound to the ribosome was compared with a cryoEM-derived model of the EF-G•GDPNP•ribosome complex revealed that ribosome-bound thiostrepton likely exhibits significant steric clash with domain V of EF-G (Harms et al., 2008). Figure 3-6 illustrates this suggested steric clash.



**Figure 3-6.** Proposed steric clash between thioestrepton and domain V of EF-G on the ribosome. Computational docking of EF-G (green) into the ribosome-thioestrepton crystal structure reveals the likelihood of significant steric clash between the bound antibiotic (cyan) and domain V of EF-G. Adapted from Harms, et al., 2008.

To test the effect of the absence of domain V on sensitivity to thioestrepton, we analyzed an available mutant of EF-G in which domains IV and V have been deleted (EF-G $\Delta$ 4,5). We subjected EF-G $\Delta$ 4,5 to the same GTP hydrolysis and binding experiments as wildtype EF-G and LepA. Interestingly, we found that EF-G $\Delta$ 4,5 behaves much differently than full-length EF-G in the presence of thioestrepton-treated ribosomes. Although the rate of ribosome-dependent GTP hydrolysis by EF-G $\Delta$ 4,5 is not as rapid as that of wild-type EF-G, this activity is completely unaffected by the addition of thioestrepton (Figure 3-2). In fact, in

dose-response experiments, EF-G $\Delta$ 4,5 shows full GTP hydrolysis activity in the presence of up to 100  $\mu$ M thiostrepton, a 500-fold molar excess of thiostrepton over ribosomes (Figure 3-3). It should be noted that above this concentration of thiostrepton, significant insoluble precipitates form and any decrease in activity is likely due to nonspecific interactions. Finally, the results of binding experiments show that EF-G $\Delta$ 4,5 is fully capable of binding to thiostrepton-bound ribosomes (Figure 3-5).

The fact that deletion of domains IV and V from EF-G ameliorates the inhibitory effects of thiostrepton supports the notion that thiostrepton functions by binding to the ribosome at the GAC and destabilizing EF-G•GTP or LepA•GTP binding to the ribosome by sterically clashing with domain V of these factors. The absence of domain IV is not likely to be the cause of the observed insensitivity of EF-G $\Delta$ 4,5 to thiostrepton for two reasons: (1) LepA does not have a domain which is homologous to domain IV of EF-G, yet is similarly inhibited by thiostrepton, and (2) no biochemical or structural evidence suggests that domain IV participates in any interactions with thiostrepton.

## Summary

It has been recently discovered that the naturally occurring antibiotic thiostrepton inhibits the growth of the deadly malarial parasite *P. falciparum*. However, the precise mechanism of action of thiostrepton is actively debated. The results presented in this work support a model for the action of thiostrepton in which the antibiotic binds irreversibly to the ribosome and destabilizes the binding of EF-G and LepA by interfering with the docking of domain V of these translation factors into a cleft between 23S GAC rRNA and ribosomal protein L11. This is in direct contrast to the predominantly accepted model which holds that thiostrepton allows factor binding but inhibits phosphate release and factor dissociation. These results may contribute in part to the development of thiostrepton as an antimalarial drug.

## **Chapter 4 - Protocol development**

### **Introduction**

The inherent complexity of translation can make experimental inquiry of ribosome function a daunting task. A reductionist approach to studying ribosome functionality in vitro can assist greatly in the simplification of experimental design and interpretation of results. However, if the components of an experimental system are overly simplified, researchers risk the possibility of not faithfully mimicking a process in a physiologically relevant manner, and erroneous results such as “false positives” can potentially result. Therefore, in the case of the ribosome, interpretations of experimental results using physiologically irrelevant ribosomal complexes must be made with caution. For example, we have confirmed that depletion of L12 from the 70S ribosome greatly diminishes GTP hydrolysis activity by the translational GTPases in the presence of vacant ribosomes (see Chapter 2), but this does not adequately explain the role of L12 in the different phases of translation such as the formation of the 70S initiation complex, translocation of tRNAs/mRNA through the ribosome, recycling of subunits, etc. In order to test the effects of alterations or deletions of translational components on actual translational processes, functional experiments must be designed to faithfully mimic the different phases of translation.

One translational process that has been well-studied is translocation. There are several examples of developed methods which enable the translocation of the tRNA-mRNA complex to be studied. One method relies on the reaction between the molecule puromycin and peptidyl tRNA bound to the ribosomal P-site (Traut and Monro, 1964). Puromycin is an A site substrate analog, and as such will cleave any peptide bound to P-site tRNA, but not A-site tRNA. If a ribosome-mRNA complex is charged with aminoacyl tRNAs in the P and A sites, the peptidyl transferase reaction spontaneously results in a deacylated tRNA in the P site and a peptidyl tRNA in the A site. EF-G-induced translocation results in the movement of peptidyl tRNA to the P site, making it puromycin reactive, which can be quantitatively measured (Sharma, 2004). This method is effective but usually requires the use of aminoacyl tRNAs in which the bound amino acid has been isotopically labeled, which can be expensive and cumbersome.

Another method of studying translocation in vitro employs the use of tRNAs which have been covalently modified with the fluorophore proflavin, and exploits the difference in fluorescence intensity between A and P site bound tRNA to monitor the translocation process (Robertson and Wintermeyer, 1987; Rodnina et al., 1997). This method allows rapid kinetic measurements of translocation using stopped-flow instrumentation, but since the fluorophore is bound directly to tRNA, the scope of this method is limited.

A third, recently developed method of studying translocation uses mRNA which has been labeled on its 3' end with a fluorescent probe. Translocation moves the fluorescent probe close to the interior of the ribosome, which results in considerable quenching of the fluorescence intensity of the fluorophore (Studer et al., 2003). This method is attractive because it is simple, sensitive, relatively non-invasive, and because any tRNAs can be used.

Another translational event that has been studied successfully in vitro is the process of ribosome recycling - the dissociation of the 70S ribosome into its 50S and 30S subunits. As discussed in Chapter 1, this process is induced by the action of ribosome recycling factor (RRF), EF-G, and IF3, in a GTP-dependent manner (Barat et al., 2007). There are two methods by which this process has been observed, sucrose gradient centrifugation and Rayleigh light scattering. The sucrose gradient method relies on the different sedimentation behavior of 70S ribosomes and its constituent subunits through sucrose gradients (Hirokawa, 2005; Kaempfer et al., 1968). In short, a sample consisting of an unknown mixture of individual ribosomal subunits and whole 70S ribosomes, or 70S ribosomes and RRF/IF3/EF-G/GTP, is layered over a sucrose gradient and subjected to ultracentrifugation. Upon subsequent fractionation of the mixture, whole 70S ribosomes can be separated from the individual subunits, and relative proportions of the two subunits and whole 70S ribosomes can then be quantified (Hirokawa, 2005). One overarching problem with this technique is that no information about the rate of subunit dissociation can be obtained. Also, the



dissociated subunits must be sufficiently stable so as not to re-associate during ultracentrifugation (Hirokawa, 2005). Another method that is capable of measuring the dissociation of 70S ribosomes relies on Rayleigh light scattering. This method takes advantage of the observation that ribosomes are large enough particles to scatter light at certain wavelengths. When ribosomes are dissociated into their subunits, the intensity of scattered light decreases proportionally (Antoun et al., 2004; Grunberg-Manago et al., 1975). This method allows for the observation of ribosome dissociation or, conversely, the association of the individual subunits to form whole 70S ribosomes, in real-time.

## **Research Aims**

The goal of this work is to develop reliable assays to measure two different phases of translation in vitro, translocation and ribosome recycling. Utilizing such assays, the physiological effect of alterations to translational components, such as the removal of ribosomal protein L12 or mutations of translation factors, could be more accurately assessed.

## Materials and Methods

### *Translocation assay*

### *Fluorescence measurements*

All fluorescence measurements were made with a spectrofluorometer (PTI international). Samples were placed in 150  $\mu$ L microcuvettes, excited at 495 nm, and emission spectra were recorded from 505-540 nm. Excitation and emission slit widths of 1.0 mm were used, and all spectra were obtained at 20°C. Before collecting emission spectra, care was taken to first incubate all samples at 20°C for 10 minutes.

### *Control samples*

A pre-translocation-like control sample consisting of ribosomes with tRNA<sup>fMet</sup> bound to the P site was prepared by incubating ribosomes (0.2  $\mu$ M) with 6-carboxyfluorescein-modified mRNA, (0.18  $\mu$ M, sequence: 5' AAG GAG GUA AAA AUG UUU GCU 3' • 6-carboxyfluorescein, Integrated DNA Technologies) and tRNA<sup>fMet</sup> (0.6  $\mu$ M) in translocation buffer (5 mM potassium phosphate, pH 7.6, 95 mM KCl, 5 mM NH<sub>4</sub>Cl, 6 mM MgCl<sub>2</sub> 0.5 mM CaCl<sub>2</sub>, 1 mM spermidine, 1 mM DTT) for 10 minutes at 37° C. A post-translocation-like control sample consisting of ribosomes with N-acetyl-Phe-tRNA<sup>Phe</sup> (NAc-Phe-tRNA<sup>Phe</sup>) bound to the P site was prepared by incubating ribosomes (0.2  $\mu$ M) with 6-

carboxyfluorescein-modified mRNA (0.18  $\mu\text{M}$ ) and NAc-Phe-tRNA<sup>fMet</sup> (0.6  $\mu\text{M}$ ), for 10 minutes at 37° C.

### *Translocation*

Pre-translocation complexes consisting of ribosomes with deacyl-tRNA<sup>fMet</sup> bound to the P site and NAc-Phe-tRNA<sup>Phe</sup> bound to the A site were formed by incubating ribosomes (0.2  $\mu\text{M}$ ) with 6-carboxyfluorescein-modified mRNA (0.18  $\mu\text{M}$ ) and tRNA<sup>fMet</sup> (0.6  $\mu\text{M}$ ) in translocation buffer for 20 minutes at 37° C, followed by addition of NAc-Phe-tRNA<sup>Phe</sup> (0.6  $\mu\text{M}$ ) and incubation for 20 minutes at 37° C. To measure EF-G-catalyzed translocation of the tRNA•mRNA complex, EF-G (1.0  $\mu\text{M}$ ) and GTP (0.5 mM) were added to pre-translocation complexes and the mixture was incubated for 20 minutes at 37° C, followed by recording of the emission spectra.

### *Ribosome dissociation measured by light-scattering*

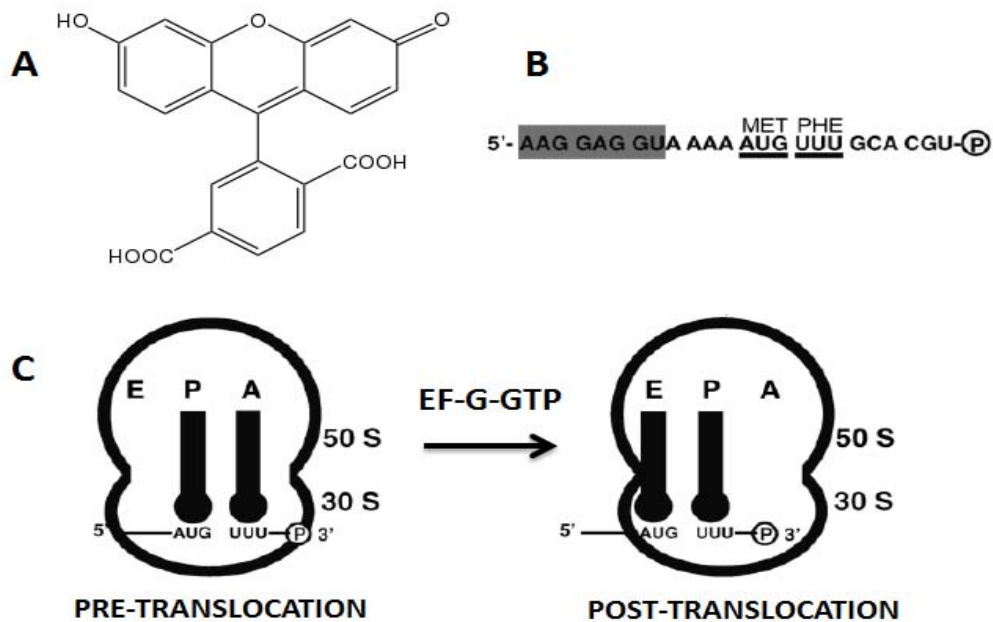
Light scattering experiments were performed at 20° C using a spectrofluorometer (PTI International). Scattering of light at 436 nm was observed by manually setting both excitation and emission wavelengths to 436 nm, and excitation and emission slit widths were both set to 1.5 mm. Before analysis, all samples were first centrifuged at 14000 rpm to remove dust particles. To measure Mg<sup>2+</sup>-induced ribosome dissociation, a 15  $\mu\text{L}$  sample consisting of 70S ribosomes in

conventional buffer (10 mM Tris-Cl, pH 7.4, 10 mM MgCl<sub>2</sub>, 80 mM NH<sub>4</sub>Cl, 0.2 mM DTT) is rapidly mixed with 135 uL of low-Mg<sup>2+</sup> buffer (10 mM Tris-Cl, pH 7.4, 1 mM MgCl<sub>2</sub>, 80 mM NH<sub>4</sub>Cl, 0.2 mM DTT) in a microcuvette, followed by immediate data collection. As a negative control, the same experiment was performed except instead using a high-Mg<sup>2+</sup> buffer (10 mM Tris-Cl, pH 7.4, 12 mM MgCl<sub>2</sub>, 80 mM NH<sub>4</sub>Cl, 0.2 mM DTT), where ribosomes are expected to remain as fully associated 70S particles.

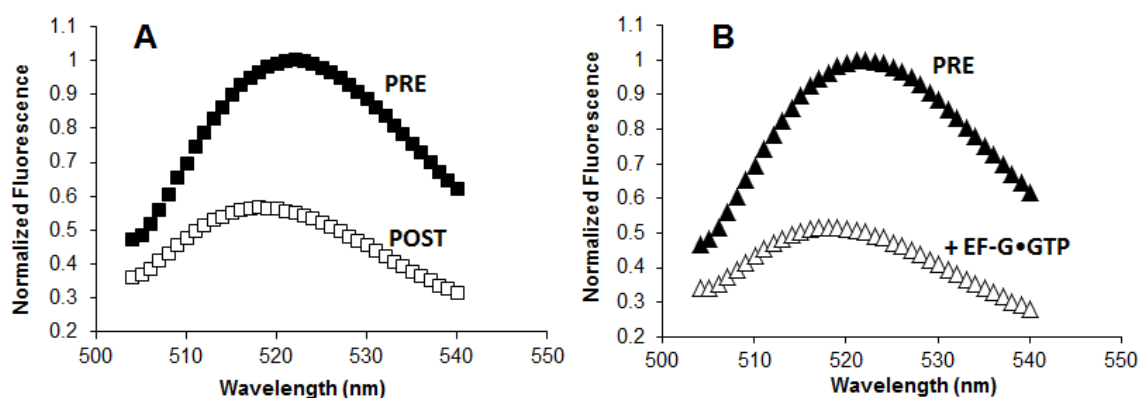
## Results and Discussion

### *Fluorescence-based translocation assay*

To measure translocation *in vitro*, we developed an assay which utilizes an mRNA which has been labeled with the fluorophore 6-carboxyfluorescein. Figure 4-1 shows a schematic representation of this assay. Figure 4-2 shows the results of experiments using a fluorescently labeled mRNA as a probe to enable observation of the process of translocation. Comparison of the emission spectra of control samples consisting of a pre- or post-translocation-like complex reveals that the artificial movement of the Phe codon from the A-site to the P-site, which also moves the mRNA-bound moiety closer to the interior of the ribosome, results in a shift in the wavelength of maximum emission intensity (from 522 nm to 518 nm), as well as an overall quenching of fluorescence intensity. Likewise, addition of EF-G•GTP to a pre-translocation complex results in identical spectral changes (Figure 4-2B), indicating that this assay is an appropriate means of observing translocation *in vitro*. The assay has been used in attempt to observe reverse translocation by LepA, but results have been inconclusive (data not shown), likely owing to the inherent difficulty in preparing stable post-translocation 70S complexes (Shoji et al., 2006), a necessary component for the functional study of LepA (Qin et al., 2006).



**Figure 4-1. Translocation assay.** (A) 6-carboxyfluorescein, the fluorophore used to label the 3' of mRNA. (B) Sequence of mRNA used. The shaded box represents the Shine-Dalgarno sequence. (C) Representation of the translocation experiment. The pre-translocation complex consists of ribosomes bound to mRNA which places tRNA<sup>Met</sup> in the P-site and tRNA<sup>Phe</sup> in the A-site. The mRNA was labeled on its 3' end with 6-carboxyfluorescein (indicated by P). Translocation, catalyzed by EF-G•GTP, moves the mRNA-tRNA complex by one codon, which positions the 6-carboxyfluorescein in a new microenvironment within the ribosome and alters its fluorescence emission spectrum. Figure adapted from Studer et al., 2003.



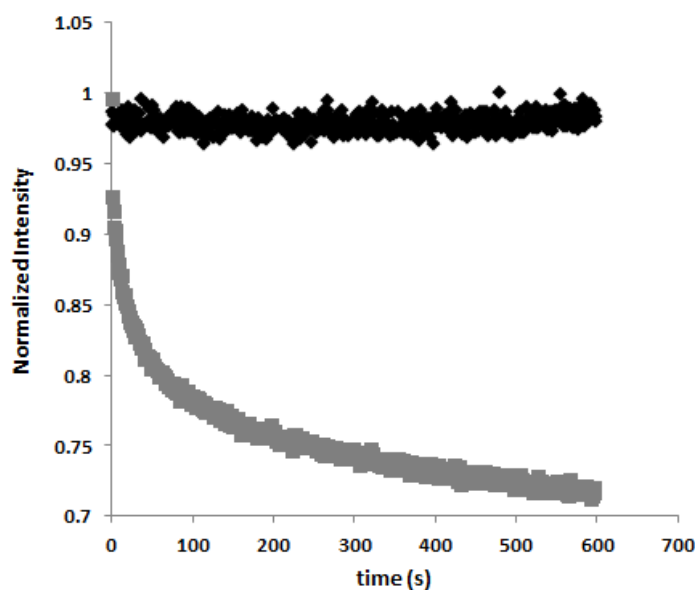
**Figure 4-2. Results of translocation assay.** (A) Control experiments. Filled squares, emission spectrum of a pre-translocation-like complex consisting of ribosomes, labeled mRNA, and tRNA<sup>fMet</sup> bound to the P site. Open squares, emission spectrum of a post-translocation-like complex consisting of ribosomes, labeled mRNA, and NAc-Phe-tRNA<sup>Phe</sup> bound to the P site. (B) Translocation. Filled triangles, emission spectrum of a pre-translocation complex consisting of ribosomes, labeled mRNA, tRNA<sup>fMet</sup> bound to the P site, and NAc-Phe-tRNA<sup>Phe</sup> bound to the A site. Open triangles, incubation of the pre-translocation complex with EF-G•GTP results in quenching of the signal intensity and a shift in the emission maximum, corresponding to the conversion to the post-translocational state.

#### *Ribosome subunit dissociation measured by light scattering*

The dissociation of the 70S ribosome into its constituent 50S and 30S subunits after the translation of a new peptide is complete is a process referred to as ribosome recycling, and it is known to involve several translation factors such as RRF, EF-G, and IF3, although details of this process remain poorly understood relative to the other phases of translation (Hirokawa, 2005). One of the objectives of this work is to develop a method of studying the dissociation of the 70S ribosome, in order to investigate the effects of changes to the ribosomal architecture, such as removal of L12 from the ribosome, on ribosome recycling. As a proof-of-concept experiment, the induced dissociation of 70S ribosomes

was attempted by subjecting a sample of ribosomes to a buffer containing a low concentration of  $Mg^{2+}$ , which has been established to cause the dissociation of ribosomal subunits (Kaempfer, 1970; Subramanian and Davis, 1970), and then observe any changes in the intensity of scattered 436 nm light. Figure 3-3 shows results from this experiment. When ribosomes are diluted into a buffer containing a high concentration of  $Mg^{2+}$ , which stabilizes the intact 70S ribosome (Hirokawa, 2005), the intensity of scattered light remains constant. However, when ribosomes are diluted into a low- $Mg^{2+}$  buffer, a steady decrease in light scattering intensity is observed, which gradually levels off to a static value. This change is interpreted as corresponding to the dissociation of 70S ribosomes into 50S and 30S subunits. The results of this experiment show that we can successfully observe the dissociation of the 70S ribosome in real-time. The development of this assay allows for future investigations of ribosome recycling.





**Figure 4-3. Observation of  $\text{Mg}^{2+}$ -induced dissociation of 70S ribosomes. Black trace, ribosomes in the presence of 12 mM  $\text{Mg}^{2+}$ . Grey trace, 70S ribosomes diluted into a buffer containing 1 mM  $\text{Mg}^{2+}$  followed by immediate data collection. Light scattering intensity was recorded for a period of 10 minutes.**

## Summary

Despite major advances in our understanding of the various steps of translation, complete and unambiguous descriptions of many mechanistic aspects of these steps remain elusive. The work presented here describes the development of assays which successfully replicate two translational processes in vitro, translocation and ribosome recycling. These experimental protocols will be used to study aspects of these processes which are currently poorly understood, such as the mechanism of LepA-catalyzed reverse translocation, the effect of L12 depletion on translocation and ribosome recycling, and the precise order of events which results in ribosome subunit dissociation.

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